Attenuation of skeletal muscle FFA-induced insulin resistance by the polyphenol resveratrol. Elucidation of the mechanisms involved

Toran Sanli, BSc

Submitted in partial fulfillment
of the degree of Master of Science, Applied Health Sciences
(Health Science)

Under the supervision of Evangelia Tsiani, Ph.D

Faculty of Applied Health Sciences,
Brock University,
St. Catharines, Ontario

DEDICATION.

I would like to thank my parents for providing me with the work ethic and support to purse my academic achievements. I would also like to acknowledge the support of my wife and fellow lab workers. You have all made my time at Brock memorable. Thank you to Dr. Evangelia Tsiani for your guidance.

ABSTRACT.

Excess plasma free fatty acids (FFA) are correlated with insulin resistance and are a risk factor for the development of type 2 diabetes. In this study we examined the effect of the polyphenol resveratrol on FFA-induced insulin resistance in skeletal muscle cells and the mechanisms involved. Incubation of L6 myotubes with the FFA palmitate significantly decreased the insulin-stimulated glucose uptake. Importantly, the effect of palmitate was ameliorated by resveratrol. Palmitate significantly increased serine phosphorylation of IRS-1 and reduced insulin-stimulated Akt phosphorylation, an effect that was abolished by resveratrol. We then investigated the effect of palmitate and resveratrol on the expression and phosphorylation of JNK, mTOR, p70-S6K, and AMPK kinases. The results demonstrated that our treatments had no effect on the expression of these proteins. However, palmitate increased the phosphorylation of mTOR and p70-S6K, whereas resveratrol abolished this effect and increased the phosphorylation of AMPK. Furthermore, all effects of resveratrol were abolished with sirtuin inhibitors, sirtinol and nicotinamide. These results indicate that resveratrol ameliorated FFA-induced insulin resistance by regulating mTOR and p70-S6K phosphorylation in skeletal muscle cells, through a mechanism involving sirtuins.

Table of Content:

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: BACKGROUND INFORMATION
INSULIN AND THE INSULIN SIGNALLING CASCADE

1.1 Insulin & Diabetes

Insulin is an essential protein hormone that regulates, metabolism, growth, and differentiation (1;2). It was first discovered at the University of Toronto in 1921 by Fredrick Banting and Charles Best (1). Insulin is produced by the β-cells of the islets of Langerhans located in the pancreas. Structurally, insulin consist of two chains of amino acids, an α-chain and a β-chain which are linked by two disulfide bonds (3).

Insulin has important biological effects on its main target tissues, skeletal muscle, liver, and adipose. It regulates carbohydrate, protein, and lipid metabolism, and cell growth and survival (3;4). Insulin is a major player in the regulation of blood glucose homeostasis. Following a meal, blood glucose levels increase, and this increase is sensed by the pancreas. The pancreas releases insulin which in turn promotes glucose uptake into skeletal muscle and fat tissue, while inhibiting glucose secretion by the liver. The net effect of these actions of insulin is to bring blood glucose levels back to normal. Figure 1 depicts these effects of insulin on its target tissue following a meal (5).

The ability of insulin to promote glucose uptake in skeletal muscle and adipose cells, while preventing glucose production by the liver is a very important physiological process (1). Lack of insulin or the inability of insulin to carry out its normal function leads to major homeostatic disturbances and to a disease known as diabetes mellitus. Diabetes is classified into two different pathologies, type 1 and type 2. Early destruction of the β-cells that produce insulin leads to insulin deficiency and the type 1 diabetes mellitus pathology (3). Individuals with type 1 diabetes require insulin injections to maintain blood glucose homeostasis. In contrast, individuals with type 2 diabetes are

characterized by compromised insulin action, and β-cell function. The factors mediating insulin resistance may be genetic and/or environmental. Obesity and reduced physical activity may also be contributors (3).

Treatment of type 2 diabetes may start with increased activity/exercise, controlled diet, and the use of pharmaceutical agents such as metformin, thiazolidinediones (TZDs), and sulfonylurease (SUs) (6). Pharmacological agents are widely prescribed and have different mechanisms of action to help regulate blood glucose homeostasis. Metformin has been reported to activate AMPK which then promotes glucose uptake into skeletal muscle cells and adipocytes (7). The TZDs (i.e. rosiglitazone) act by binding to the nuclear receptor peroxisome-proliferator-activator receptor γ (PPARγ), which increases insulin sensitivity and decreases glucose production by the liver (8). Sulfonylureas act by stimulating the β-cells of the pancreas to secret more insulin. This occurs when the drug binds to the ATP-dependent K^+ channel of the β-cell, causing depolarization on the β-cell membrane, thereby releasing insulin into the blood stream (6).

1.2 Glucose Transport Proteins

Due to size and its hydrophobic properties, glucose can not freely pass through the cell membrane lipid bilayer. Therefore glucose, with a molecular weight of 180 is transferred through the plasma membrane by facilitated diffusion. A family of glucose transport proteins known as the GLUT family allow facilitated entry of glucose into the cell (1). The GLUT family consists of at least 12 different members, which are structurally similar to each other. All GLUT proteins have a transmembrane region that makes up the majority of the protein. They also contain a less conserved, asymmetric, exoplasmic region. Within the transmembrane regions are 12 membrane-spanning-helices,

which contain an N-terminal segment and a C-terminal segment in the cytoplasm and an extracellular N-linked oligosaccharide structure (9;10).

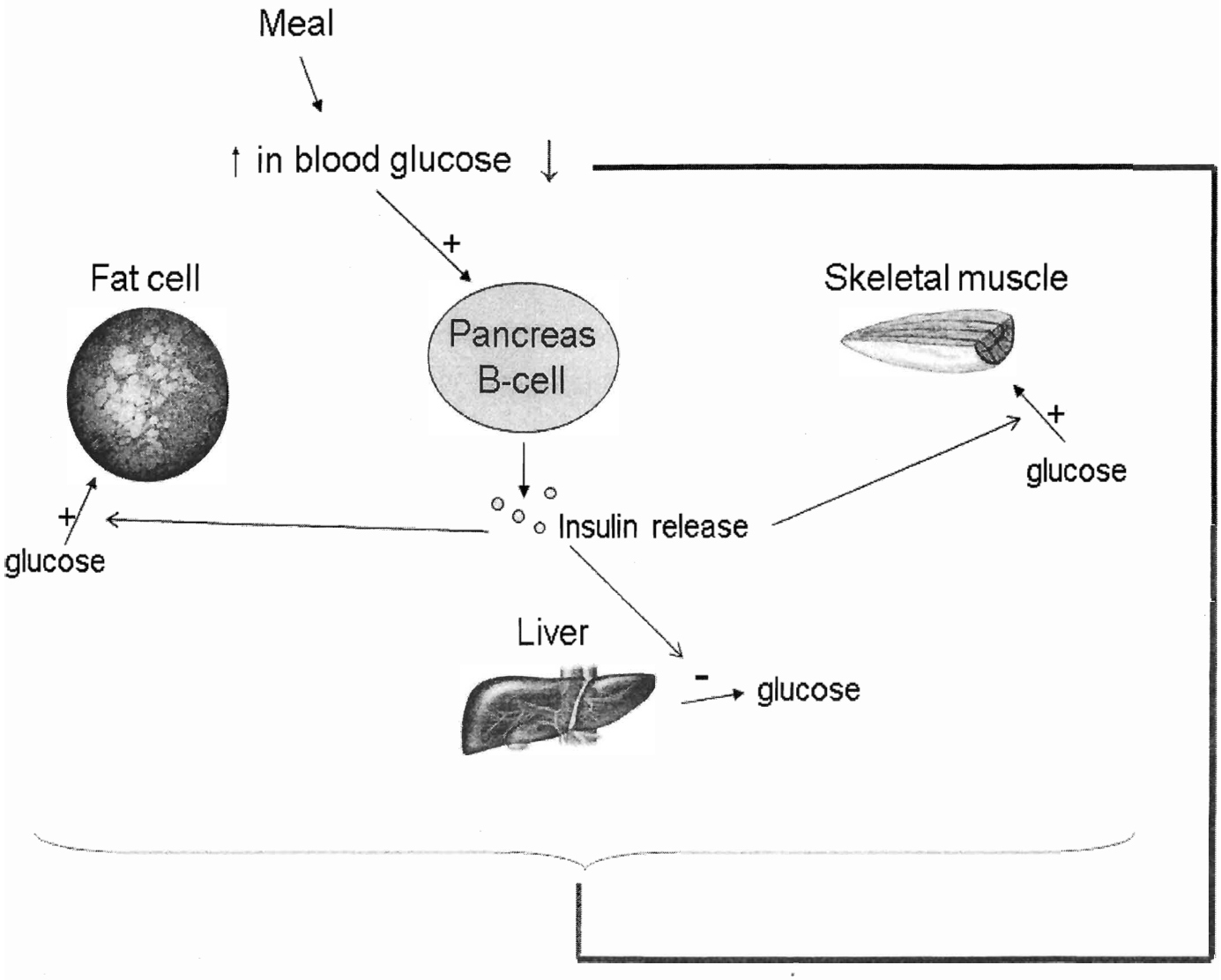


Figure 1: The effect of insulin on its target tissues to regulate blood glucose homeostasis.

The naming of each GLUT isoform follows its chronological cloning. The GLUT proteins were isolated and identified by cDNA cloning experiments using reverse

transcription polymerase chain reaction (RT-PCR) (9).GLUT1 was the first GLUT to be cloned from brain tissue. It is ubiquitously expressed in the body. GLUT2 is expressed in the liver, intestine, and kidneys. GLUT3 is present in the placenta, testis, and neurons (11). GLUT4 is the major insulin-responsive glucose transporter and is expressed in skeletal muscle, heart and fat tissue (9). The GLUT1, GLUT2, GLUT3 and GLUT4 proteins have been extensively studied, while the remaining GLUT6-12 proteins are proteins that have no known function (9).

Table 1 lists all of the glucose transporters identified so far, along with each transporter tissue expression and known function (9). Apart from differences in tissue distribution, the GLUT family members have different affinities for glucose. For example, GLUT1 has a Km of 20mM for glucose, GLUT2 has a Km of 42mM, GLUT3's Km for glucose is 10mM, GLUT4 has a Km of 2-10mM, and GLUT5 does not have significant affinity for glucose, but rather a high affinity for fructose. The differences in glucose affinity by each of the GLUT family members are physiologically important in each of the tissues they are expressed, and play a role in the maintenance of whole body glucose homeostasis (9;12).

In the absence of insulin stimulation in muscle and fat cells, GLUT4 is localized in intracellular membrane vesicles, also known as GLUT4 storage vesicles (GSV's) (1). When insulin binds and activates its receptor, it causes receptor autophosphorylation on tyrosine residues and increased tyrosine kinase activity, which in turn will phosphorylate insulin receptor substrates (IRS). Once phosphorylated, IRS will allow phosphatidylinositol 3-kinase (PI3-K) to bind to it, triggering PI3-K activation. PI3-K in the active state will cause activation of phosphoinositide dependent kinase 1 (PDK1),

which will subsequently phosphorylate Akt. This sequence of events will cause the GSV's to translocate from there cytoplasmic location to the cell surface (as seen in figure 2) (1;10). Once insulin is removed GLUT4 proteins cycle back to their storage sites (13).

Table 1: GLUT protein family expression in specific target tissues (9)

Tissue-specific expression of the GLUT proteins		
Protein	*Expression*	*Function*
GLUT1	All tissue	Basal uptake
GLUT2	Liver, pancreas	Glucose sensing
GLUT3	Brain	Complements GLUT1
GLUT4	Muslce, fat, heart	Insulin responsive
GLUT5	Intestine, testis, kidney, erythrocytes	Fructose transport
GLUT6	Spleen, leukocytes, brain	Fructose transport
GLUT7	Liver	Fructose transport
GLUT8	Testis, brain	Fructose transport
GLUT9	Liver, kidney	Fructose transport
GLUT10	Liver, pancreas	Fructose transport
GLUT11	Heart, muscle	Fructose transport
GLUT12	Heart, prostate	Fructose transport

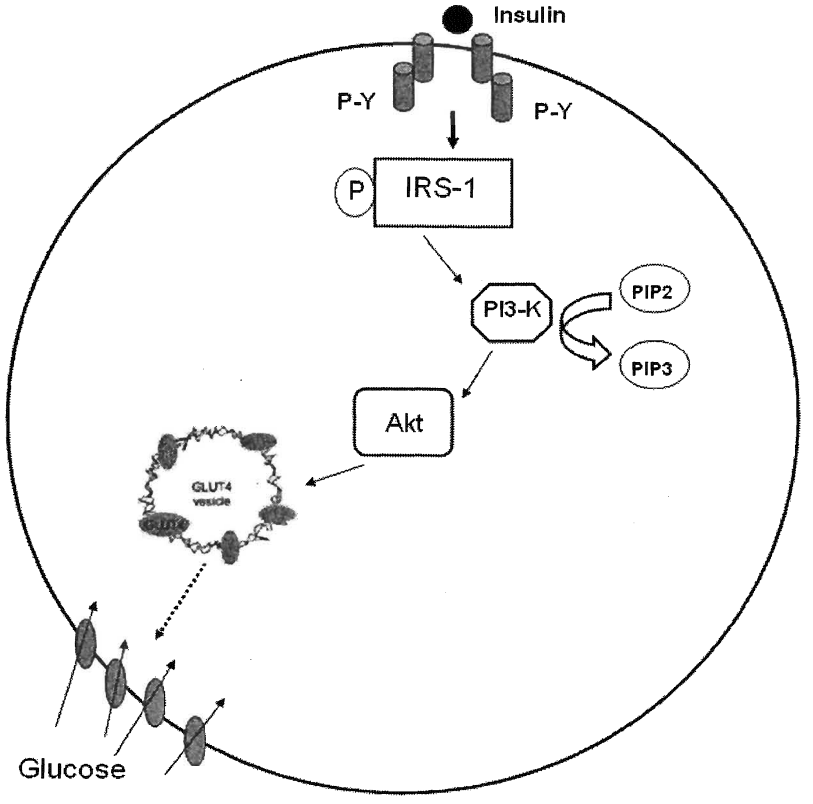


Figure 2: Insulin signalling pathway leading to translocation of GLUT4 to the plasma membrane in skeletal muscle

The importance of GLUT4 for skeletal muscle glucose transport is evident by studies using mice with specific GLUT4 knockout strategies. Muscle specific GLUT4 knockout mice (muscle-G4KO) exhibit a 95% reduction in GLUT4 expression, a 40-50% decrease in basal glucose transport in soleus skeletal muscle, and are insulin resistant since they demonstrate complete abolishment of insulin-stimulated glucose uptake in skeletal muscle (14). Furthermore, it has also been demonstrated that the pathological consequences of GLUT4 homozygous allele knockouts are growth retardation, enlarged hearts (3-3.5 times greater that control mice), and absence of white adipose tissue (15).

1.3a The Insulin Receptor and the Insulin Signalling Cascade

Insulin binding to its receptor employs many different intracellular molecules/proteins that relay the insulin signal downstream within the cell, leading to an insulin-induced cellular response. The insulin receptor belongs to a large family of cell surface receptors possessing intrinsic tyrosine kinase activity (16).

Structurally, the insulin receptor is composed of two extracellular α-subunits, and two transmembrane β-subunits linked by disulfide bonds, as seen in figure 3. The α-subunit is composed of 723 amino acids and contains the insulin binding domain. The β-subunit is made up of 620 amino acids and is composed of three compartmentalized regions: the extracellular, transmembrane, and cytosolic domains (17). The cytosolic tyrosine kinase domain of the β-subunit has an ATP binding consensus sequence and three clusters of tyrosine residues that can be phosphorylated in response to insulin (17). These three clusters are found in the juxtamembrane domain that is proximal to the cell surface, a regulatory region located in the center of the β-subunit, and the COOH-tail region (16).

Insulin binds to the extracellular α-subunit of the insulin receptor, which leads to conformational change. This results in multisite autophosphorylation of the tyrosine residues on the β-subunit of the receptor, leading to increased tyrosine kinase activity (16). There are a total of 13 tyrosine residues in the β-subunit of the insulin receptor (17). The individual sites of autophosphorylation have been recognized and correlated with the tyrosine kinase activity and biological action of the receptor. The key tyrosine (Y) residues that are phosphorylated on the β-subunit are Y953, Y960, and Y972 of the juxtamembrane region, Y1146, Y1151, and Y1152 of the catalytic domain, and Y1316,

and Y1322 of the COOH-terminal domain. The juxtamembrane region account for approximately 15% of total autophosphorylation activity of the receptor, while the catalytic domain accounts for 50%, and the COOH-terminal accounts for 30-35% (17).

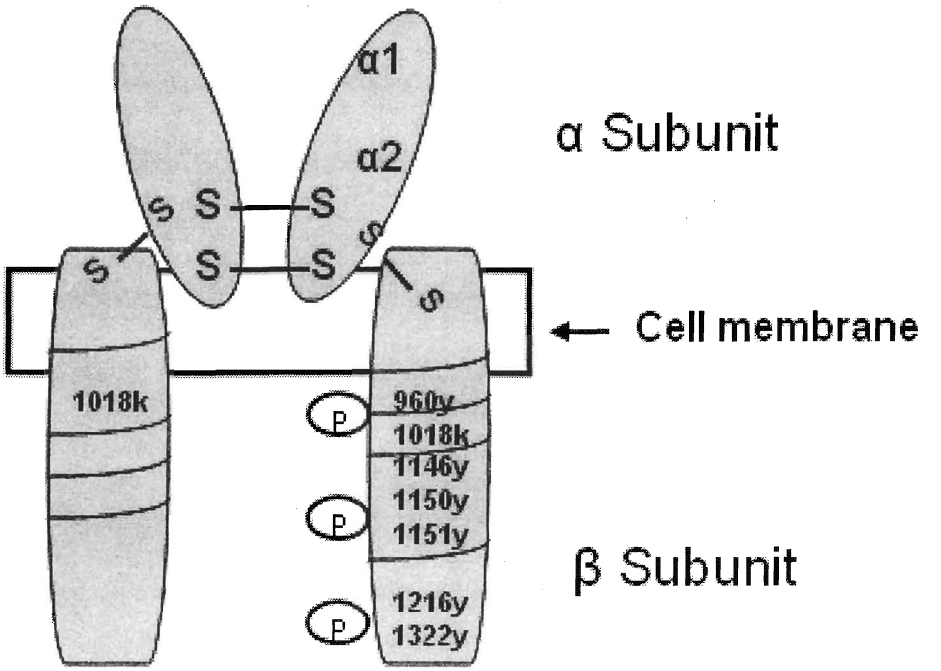


Figure 3: Schematic representation of the insulin receptor.

Point mutations that substitute phenylalanine for a specific tyrosine residue in the β-cytosolic domain of the insulin receptor has shown decreased tyrosine kinase activity (17). The most important tyrosine residues appear to be Y1146, Y1151, and Y1152 in the catalytic domain of the insulin receptor. When these tyrosine residues were substituted with phenylalanine there was significant inhibition of insulin action (17). Furthermore, when all the tyrosine residues in the three domains of the β-subunit of the insulin receptor were substituted for phenylalanine, the biological effects of insulin were completely abolished.

1.3b Insulin Receptor Substrates

Insulin receptor tyrosine kinase (RTK) activation leads to phosphorylation of insulin receptor substrates (IRS). The IRS are a family of cytoplasmic proteins that contain 22 prospective tyrosine phosphorylation sites that act as recognition sites for cellular substrates containing specific SH2 domains (16). All of the IRS proteins share a common NH_2-terminal pleckstrin homology (PH) domain, with subsequent phosphotyrosine binding (PTB) domain, and a domain of varying length containing numerous potential tyrosine phosphorylation sites (16;18).

Presently, there are nine members of the IRS family, four IRS proteins (IRS1-4), three Src-homology collagen (SHC) proteins, Grb2 associated binder-I (Gab-I), and $p62_{dok}$ (19). The IRS-1 (180kDa) and IRS-2 (185kDa) have been extensively studied, and their role in insulin-stimulated glucose uptake and glucose metabolism has been established. Once IRS-1 is phosphorylated, it acts as a docking protein for other SH2 domain-containing proteins. For example, the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3-K) binds to IRS-1, and this leads to the activation of its catalytic subunit propagating the insulin signal downstream. The IRS-3 protein is expressed mainly in adipose tissue, and does not play a role in glucose uptake. Similarly, IRS-4 is not involved in glucose homeostasis, but is highly expressed in the neuroendocrine tissue (19). The functions of IRS-3 and IRS-4 are still being elucidated.

To evaluate the involvement of IRS-1 in glucose uptake and metabolism, global gene IRS-1 knockout mice were examined (20). Mice with IRS-1 knockouts demonstrated abnormal glucose tolerance and low birth weight, up to 40-60% less than their wild-type mice counterpart (20). However, these mice did not develop diabetes due

to the development of β-cell hyperplasia (20). A similar study by Araujo et al, 2005 showed similar results in Wistar rats that experienced short-term IRS-1 inhibition via an antisense oligonucleotide (IRS-1AS) (21). This study showed a 40% decrease in Akt phosphorylation and a 30% decrease in ERK tyrosine phosphorylation in the skeletal muscle of IRS-1AS rats. In addition, the expression of IRS-1 in skeletal muscle was reduced by 75% (21).

By contrast, IRS-2 is specific to insulin action in the liver, as well as β-cell growth and survival. IRS-2 knockout mice exhibit only a small reduction in weight (10%), but develop diabetes early in life due to poor insulin secretion (20). Conversely, the role of IRS-3 and IRS-4 in glucose homeostasis is negligible when compared to the IRS-1 and IRS-2 isoforms. In fact, IRS-3 and IRS-4 knockout mice appear similar to their wild-type counterparts with normal insulin sensitivity (20). This evidence demonstrates that the IRS-1 and IRS-2 proteins play a vital role in the regulation of the insulin signalling cascade.

1.3c The PI3-K Pathway

The phosphatidylinositol 3-kinase (PI3-K) protein was initially recognized as a signalling molecule responsible for the phosphorylation on the D-3 position of the inositol head group of phosphoinositides (19). This family of proteins consists of 14 members separated into four classes of which class I, II and III are lipid kinases, and class IV are related protein kinases (22). In mammals both class I and class II PI3-K proteins have been discovered. However, only the heterodimeric p85/p110 PI3-K of the class I family of proteins is stimulated by insulin (19).

The class I family of PI3-K proteins consists of a p85 regulatory subunit that contains SH2 domains, and a p110 catalytic subunit responsible for its activity. This class is also further subdivided into class 1A and class 1B. Sub-class 1A incorporates the p110α and p110β catalytic subunit, while sub-class 1B contains the p110γ catalytic subunit (22). There are many isoforms of the regulatory p85 subunit as well. Each p85 subunit contains two SH2 domains, which bind to tyrosine phosphorylated motifs on IRS proteins and growth factor receptors (19). The isoforms 85α, p55α, p50α, p85β, and p55γ may all be bound by class 1A catalytic subunits. Alternatively, the p110 catalytic subunit may be bound by the class 1B catalytic subunit (19). Also, the proteins PI4-K and PI5-K have been shown to share a similar homology to PI3-K, but have no function in glucose metabolism.

Once phosphorylated, the IRS proteins act as docking sites for the regulatory subunit (p85) of PI3-K that will be recruited and bind to IRS via its SH2 domain. The interaction of the IRS protein and the p85 subunit of PI3-K will in turn activate the catalytic subunit (p110) of PI3-K. These steps lead to PI3-K recruitment to the plasma membrane, wherein the lipid kinase will generate a pool of lipid messenger, known as phosphatidylinositol (3,4,5) triphosphate (PIP3). This event will initiate a series of steps that include the recruitment/activation of effector proteins that carry PIP3 binding domains, such as phosphoinositide dependent kinase-1 (PDK1). PDK1 has a pleckstrin-homology (PH) domain that recognizes various phosphoinositides generated by PI3-K activation (23). PH domains bind with different affinities to the phosphoinositides, which in turn interact with the PH domain of the serine/threonine kinase Akt, causing it to become activated (23).

Two of the widely used PI3-K inhibitors are wortmannin and LY294002. Wortmannin is a naturally occurring metabolite of *Penicillium funiculosum,* while LY294002 was derived from the flavonoid quercetin (22). PI3-K inhibition by wortmannin is obtained *in vitro* at a concentration of 10nM in skeletal muscle, and works at higher concentration to exert its effect on PI3-K related proteins such as mTOR and DNA-PK. Similarly, inhibition of PI3-K with LY294002 is observed around 50μM *in vitro* (24). Initial studies using the PI3-K inhibitors show that PI3-K plays a vital role in the insulin stimulated glucose uptake. Furthermore, mice lacking PI3-K expression in skeletal muscle suffered from impaired glucose metabolism (22). This study demonstrated that PI3-K is a key player in mediating the effect of insulin to promote glucose uptake into skeletal muscle tissue.

1.3d The Akt/PKB Pathway

Akt/PKB (protein kinase B) is a 56 kDa insulin-activated protooncogen that encodes a serine-threonine kinase that is the downstream target of PI3-K (25). Akt was originally identified as a protein kinase with similar homology to the protein kinases A and C, and was thus termed PKB. In addition, Akt is the cellular homologue of the viral oncoprotein v-Akt, and is also referred to as c-Akt (26). There are three different isoforms of Akt, which are known as Akt1, Akt2, and Akt3. Akt1 is the most ubiquitously expressed isoform in mammalian tissues. Akt2 is also highly expressed in the majority of tissues and organs, at levels usually lower that Akt1. Akt2 is predominantly found in insulin target tissues, such as adipose, liver, and skeletal muscle cells. On the other hand, Akt3 is expressed in low levels in mammalian cells, except for in the testes and brain (25).

All three isoforms are similar in size and structure and contain an amino-terminal pleckstrin homology (PH) domain, a C-terminal hydrophobic motif (HM), and a kinase domain (25). The PH domain facilitates the binding of Akt to the 3-phosphoinositides and PIP_3 that are created by PI3-K (27). There are two specific sites, one in the kinase domain (Thr^{308}) and the other in the C-terminal regulatory region (Ser^{473}) that need to be phosphorylated for full activation of Akt (27). This kinase is cytosolic in unstimulated cells, and some of it translocates to the plasma membrane upon PI3-K activation, where it in turn becomes activated by PI3-K. In the active state, Akt will detach from the plasma membrane and migrate through the cytosol and into the nucleus (26). How this mechanism of action of Akt migration into the nucleus is achieved is currently unclear.

Akt can become stimulated by insulin and other growth factors, such as platelet derived growth factor (PDGF), and epidermal growth factor (EGF) (28). Specifically, in skeletal muscle and adipocytes, Akt is stimulated by insulin which leads to GLUT4 translocation and increased glucose uptake in mammalian cells. Apart from glucose uptake, Akt also has many mitogenic functions within the cell (26). It may help regulate cell size and cell survival, as well as control cellular metabolism as seen in figure 4 (28).

To address Akt's role in glucose uptake, studies have used a constitutively active mutant of Akt in 3T3-L1 adipocytes (28). Overexpression of a constitutively active mutant of Akt (Akt-myr) resulted in a significant increase in GLUT4 translocation to the cell membrane and increased glucose uptake, even in the absence of insulin. In addition, other studies have demonstrated that Akt is co-localized with GLUT4-containing vesicles in 3T3-L1 adipocyte cells (29). Also, use of a dominant inhibitory mutant of Akt that is kinase-deficient, resulting from the substitution of an alanine for lysine at the 179 residue

in the canonical ATP-binding domain, failed to cause GLUT4 translocation to the plasma membrane of adipocytes, even in the presence of insulin-stimulation (28). A similar study by Wang et al, 1999 investigated the role of Akt in GLUT4 translocation and glucose uptake in L6-GLUT4myc skeletal muscle cells (30). A constitutively active Akt protein created by fusion of a viral Gag protein at its N terminus (GagAkt), increased the cell surface density of GLUT4myc compared to control cells (30). Furthermore, the study also employed the use of a kinase-inactive phosphorylation-deficient Akt construct (substitution of alanine for the lysine at position 179 of Akt) to examine the function of Akt in GLUT4 translocation and insulin-stimulated glucose uptake. There was a marked inhibition of GLUT4 translocation to the plasma membrane and abolished insulin-stimulated glucose uptake in L6 myotubes that had the dominant-negative Akt mutant (30). Taken together, these studies demonstrate that glucose uptake via GLUT4 translocation in adipose and skeletal muscle tissue is dependent on Akt function. Figure 4 shows some of the downstream Akt effects in mammalian cells.

In vivo approaches using knockout mice (Akt1-/-, and Akt2-/-2) have been taken to examine Akt function. Akt1-/- knockout mice showed defects in both fetal and postnatal growth that carry on into adulthood (31). However, these mice have normal glucose tolerance and insulin sensitivity. In contrast, Akt2-/- mice exhibited insulin resistance and became diabetic (32). These studies demonstrated the different physiologic roles the Akt isoforms. In addition to *in vivo* reports, some studies have used small interfering RNA (siRNA) to deplete both Akt1 and Akt2 is 3T3-L1 cultured adipocytes (33). Loss of Akt1 alone caused slight impairment of insulin-stimulated glucose transport and had no effect on glycogen synthase kinase (GSK)-3 phosphorylation. Conversely, a

70% reduction of Akt2 levels alone inhibited almost half of the insulin-stimulated response, indicating that Akt2 is required for normal insulin-responsiveness in 3T3-L1 adipocytes (33).

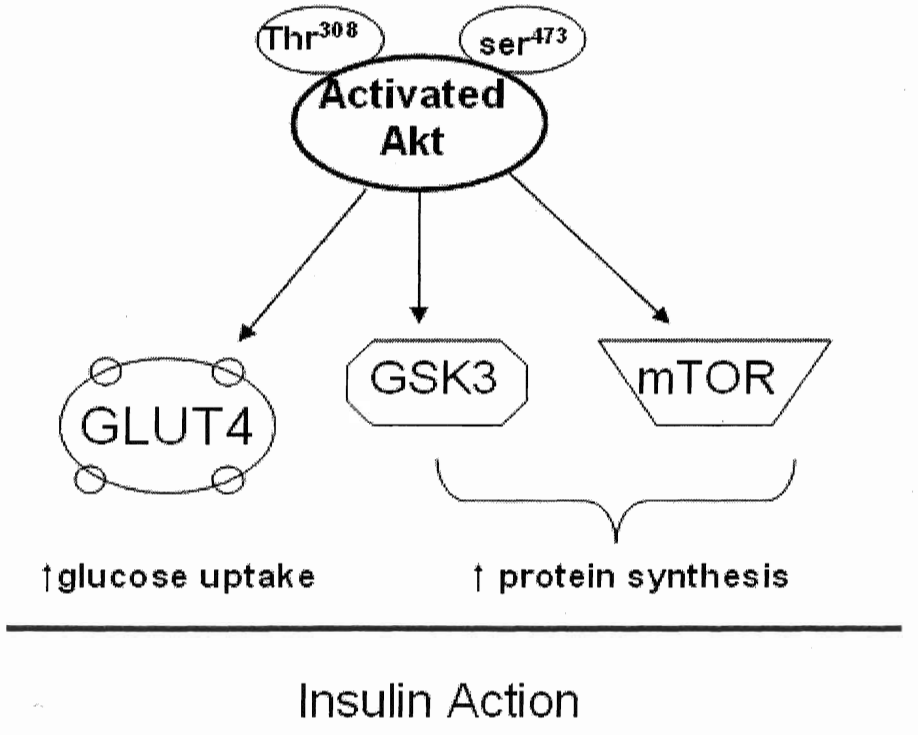


Figure 4: The functions of Akt in the insulin signalling cascade in mammalian cells.

INSULIN RESISTANCE IN SKELETAL MUSCLE

1.4a Insulin Resistance in Skeletal Muscle Cells

Skeletal muscle is quantitatively the most important target tissue of insulin, and defects in the mechanisms regulating insulin-stimulated glucose transport in skeletal muscle will lead to insulin resistance (34;35). Decreased insulin-stimulated glucose uptake may be due to decreased expression of the insulin receptor, reduced tyrosine phosphorylation of the insulin receptor or IRS-1 due to decreased tyrosine kinase activity of the receptor. Phosphorylation and activation of downstream proteins including Akt as

well as GLUT4 translocation to the plasma membrane may also be diminished (36). It has been established that serine phosphorylation of IRS-1 leads to impairment in the insulin signalling cascade and a significant reduction in insulin-stimulated glucose uptake (34;37;38).

Insulin resistance is highly correlated with obesity and is a risk factor for the development of type 2 diabetes, metabolic syndrome, and cardiovascular disease (36). It has been shown that insulin-induced glucose uptake in skeletal muscle is significantly reduced in obese individuals and patients with type 2 diabetes (36). Moreover, decreased expression of IRS-1 and low levels of tyrosine phosphorylation have been found in 30% of subjects at high risk for type 2 diabetes, such as obese individuals and subjects that are first-degree relatives of type 2 diabetics (39). Several signalling molecules, including GSK3 (40), c-Jun N-terminal kinase (JNK) (41;42), PKC's (43), NFκβ (44), mTOR (45;46), and p70 S6K (47;48) have been implicated to mediate insulin resistance. All of the above molecules have been shown to induce insulin resistance by serine phosphorylation of the IRS-1 protein, leading to decreased insulin-stimulated glucose uptake in skeletal muscle and adipose cells, as seen in figure 5 (36). There are many other studies indicating that increased saturated free fatty acids (FFA) levels leads to insulin resistance in skeletal muscle by mechanisms involving the above mentioned signalling molecules (41;43-45;47;49;50). In the following sections a review is presented of the role of these signalling molecules in mediating insulin resistance.

1.4b Insulin Resistance and Serine (ser) Phosphorylation of IRS-1

The notion of serine phosphorylation of IRS-1 impairing insulin action was first demonstrated 14 years ago when researchers used the serine phosphatase inhibitor

okadaic acid in skeletal muscle cells and 3T3-L1 adipocytes (51). Okadaic acid significantly diminished the effect of insulin on GLUT4 translocation and glucose transport in these respective tissues, by decreasing tyrosine phosphorylation on IRS-1, and downstream PI3-K activation. It was later discovered that okadaic acid specifically increases serine phosphorylation of IRS-1 (39;49). It was shown that prolonged hyperinsulinemia in skeletal muscle cells and adipocytes also induce serine phosphorylation of IRS-1. This indicates that there may be a negative feedback mechanism in place that uncouples the IRS-1 protein from its upstream/downstream members, and blocks insulin signal transduction under physiological conditions (50).

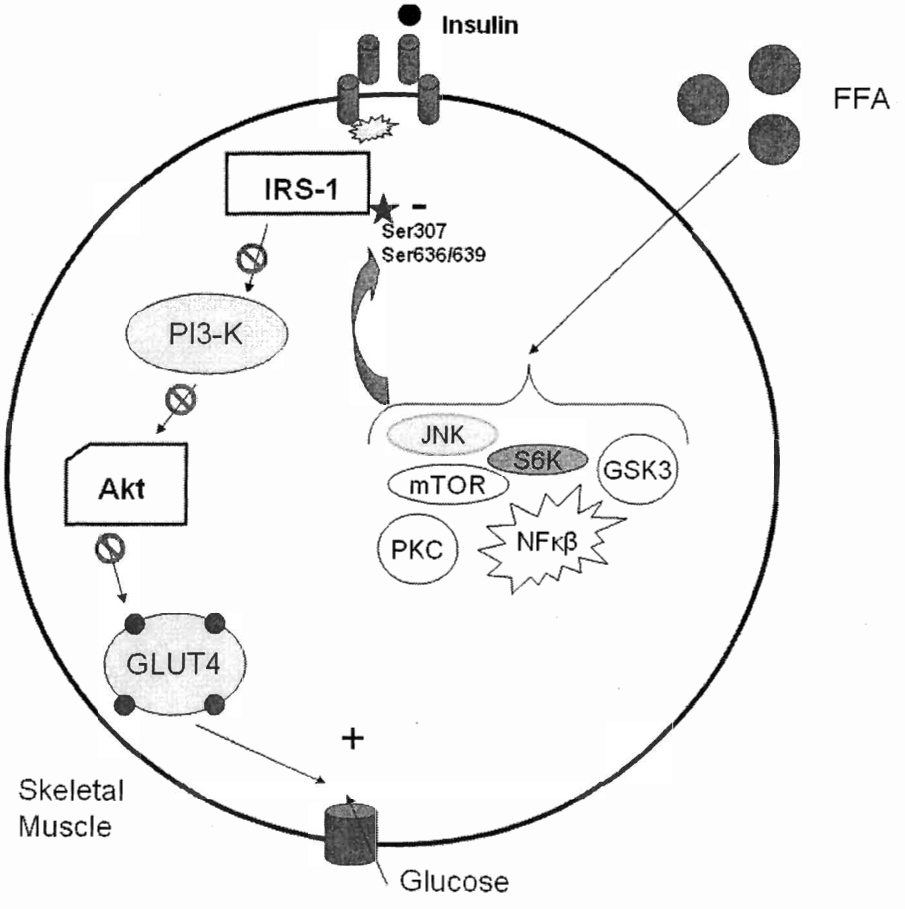


Figure 5: Proposed mechanism of FFA-induced insulin resistance in skeletal muscle cells

In vitro L6 myotubes have often been employed to study the mechanism behind serine phosphorylation of IRS-1 and skeletal muscle insulin resistance (52). This is because they are the best available *in vitro* skeletal muscle system, and are insulin responsive. However, these cells have a high basal serine phosphorylation of IRS-1 protein when compared to intact human skeletal muscle (52).

The increased serine phosphorylation of IRS-1 is clearly linked to decreased tyrosine phosphorylation, leading to reduced glucose uptake (53). Many cytokines and protein kinases (as previously mentioned) not only induce serine phosphorylation of IRS-1 on its serine residue, but will simultaneously diminish insulin-induced tyrosine phosphorylation of IRS-1. Hyperphosphorylated IRS-1 on serine residues makes a poor substrate for the insulin receptor, thereby inhibiting its downstream effects. For example, diabetogenic stimuli may activate serine/threonine protein kinases or inactivate serine/threonine phosphatases, leading to increased serine phosphorylation of IRS-1, as seen in figure 5 and table 2 (37). In rodent models of obesity, inhibiting serine phosphorylation of IRS-1 leads to increased insulin sensitivity in parallel with correction of the impaired insulin-induced tyrosine phosphorylation of IRS-1 (37;41;47;54).

Several serine residues on IRS-1 have been identified and linked to decreased insulin-stimulated IRS-1 function and specifically IRS1-PI3K association, downstream Akt activation, and glucose uptake (39). Ser^{307} is located at the end of the phospho-tyrosine binding (PTB) domain of IRS-1, which modulates the interaction of IRS-1 with the insulin receptor (55). The association of IRS-1 with the insulin receptor is significantly decreased once IRS-1 becomes phosphorylated on ser^{307} by stimuli such as JNK, mTOR, p70 S6K, or FFA (39;55). In addition, it has been shown *in vivo* that

decreased phosphorylation at ser^{612} by MAPK correlates with enhanced insulin-stimulated tyrosine phosphorylation of IRS-1 (2). Similarly, the $ser^{636/639}$ residues have been implicated in inhibiting IRS-1 activity and are increased in rats fed a high fat diet *in vivo* (56). Finally, increased ser^{1101} phosphorylation by PKCθ has been implicated in blocking IRS-1 tyrosine phosphorylation and downstream activation of Akt in C2C12 skeletal muscle cells (43;57). Table 2 shows the various serine phosphorylation sites of IRS-1 and how they regulate insulin signalling.

Table 2: The known sites of IRS-1 serine phosphorylation and the kinases involved

Serine Residue	Stimuli	Tissue	Effect on Insulin Signalling	Reference
307	FFA, okadaic acid, TNFα, mTOR, p70 S6K, JNK	Muscle Liver Fat	Decreased action	Aguiree et al 2000 (51) Gual et al 2004 (37) Brustel et al 2003 (47)
612	ERK, mTOR	Muscle Liver	Decreased action	Taniguchi et al 2006 (2)
636/639	FFA, mTOR, p70 S6K	Muscle Liver Fat	Decreased action	Le Bo et al 2007(52) Gual et al 2004 (37)
1101	PKCθ, FFA	Muscle Liver	Decreased action	Li et al 2004 (41) Tremblay et al 2007 (53)

1.4c The GSK3 Pathway

Glycogen synthase kinase-3 (GSK3) is a 51 kDa serine/threonine protein kinase that plays a role in the regulation of glycogen synthesis (40). GSK3 phosphorylates and inactivates glycogen synthase and is involved in the PI3-K/Akt survival pathway (40). In

addition, GSK3 has been established to regulate glucose homeostasis and has been implicated in the development of insulin resistance (58).

There are two different isoforms of GSK3, know as GSK3α and GSK3β which have a high degree of homology in structure and biological function (59). Both isoforms of GSK3 have an inhibitory serine phosphorylation site (ser^{21} for α, and ser^{9} for β), and a catalytic tyrosine activation site (Y279 for α, and Y216 for β) (40). GSK3 is almost always constitutively active, and Akt or extracellular stimuli can induce serine phosphorylation of GSK3 to inhibit its activity (40).

Studies in 3T3-L1 adipocytes has demonstrated that GSK3 increases serine phosphorylation of IRS-1 and can attenuate the insulin-stimulated tyrosine phosphorylation on the insulin receptor and IRS-1 (60;61). Furthermore, an association between GSK3 and insulin resistance has also been found in reported cases of type 2 diabetes (58). The expression and activity of both GSK3α and GSK3β is elevated in the skeletal muscle tissue of poorly controlled type 2 diabetic patients (58). In addition, there is an inverse relationship to insulin action and increased GSK3 levels in type 2 diabetic patients that underwent hyperinsulinemic/euglycemic clamp tests (58). Taken together, GSK3 is a key player in the modulation of glucose homeostasis, and may mediate the development of insulin resistance.

1.4d Insulin Resistance and c-Jun N-terminal Kinase (JNK)

JNK is a serine/threonine kinase that is also known as stress activated protein kinase (SAPK), and is a member of the mitogen activated protein kinase (MAPK) family (54). There are three different isoforms in the JNK family, which are denoted JNK1 (46kDa), JNK2 (54kDA), and JNK3 (54kDA). Of these members, JNK1 and JNK2 are

ubiquitously expressed, whereas JNK3 is expressed mainly in the heart, testis, and the brain (54). JNK1 is the isoform that has been speculated to play a role in the pathology behind obesity and insulin resistance. When activated, JNK forms a dimer that can translocate into the nucleus where it regulates the expression and phosphorylation of transcription factors such as c-Jun, which in turn modulate gene transcription (42).

JNK, can be activated by many environmental stresses, such as radiation, growth factors, cytokines like tumor necrosis factor alpha (TNFα) or interleukin-1β (IL-1β), and elevated FFA (54). There is evidence found in high fat fed or genetic knockout mice models that implicate JNK as a mediator of insulin resistance (62). It was previously demonstrated that rats fed a diet high in saturated fat have increased JNK activity. This increase in JNK activation was correlated with ser^{307} phosphorylation of IRS-1 in liver and skeletal muscle (54). These results were compared to rats fed a control diet for 10 or 30 days, with insulin resistance being confirmed by hyperinsulinemic-euglycemic clamp procedures in both hepatic and skeletal muscle tissue. Only the rats that had developed insulin resistance by being fed a high fat diet showed increased JNK phosphorylation.

Also, it has been recently demonstrated that JNK-interacting protein I (JIP1), which binds components of the JNK signalling pathway, is required for JNK activation and inhibition of IRS function in adipose tissue of obese mice (39). In epididymal fat pads from JIP1-deficient mice, insulin-stimulated tyrosine phosphorylation of IRS-1 was increased and the inhibitory phosphorylation of IRS-1 on ser^{307} residue was decreased (39). Moreover, sustained activation of JNK was seen in primary mouse hepatocytes and pancreatic β-cells of fat diet-induced insulin resistant mice and streptozotocin (STZ)-induced diabetic mice. Conversely, mice with a targeted mutation at the JNK1 locus that

abolished JNK expression, proved to be resistant to high fat-induced insulin resistance (62). The study by Solinas et al 2006, was in adherence with previous findings, in that the major mechanism behind JNK-mediated insulin resistance is through the ser^{307} phosphorylation of IRS-1 (55).

Studies have also addressed the different roles between JNK1 and JNK2 in mediating high fat diet-induced insulin resistance and obesity in mice (54). It has been well documented that JNK1-/- or JNK1 mutant mice have improved insulin sensitivity and protection from high fat diet-induced insulin resistance in liver tissue. Because JNK2-/- mice did not experience any significant changes in insulin sensitivity, JNK2 was ruled out as a key regulator in obesity and type 2 diabetes (42) Interestingly, JNK2 but not JNK1 deficiency has been reported to provide protection against atherosclerosis and type 1 diabetes in mice. Thus, both isoforms of JNK play a role in mediating some aspect of the diabetes pathology. Furthermore, studies *in vitro* have shown that JNK1 and JNK2 exhibit similar affinity and activity towards ser^{307} phosphorylation of IRS-1 in HEK293T hepatocyte cells (42).

Antidiabetic medication such as rosiglitazone has been shown to inhibit JNK activation *in vivo*, and normalizes blood glucose homeostasis in insulin resistant mice (63). High fat diet-induced obese mice that were given rosiglitazone demonstrated marked JNK inhibition and reduced insulin resistance (64). Furthermore, JNK is involved in the apoptosis of pancreatic β-cells induced by the IL-1β cytokine, which is prevented by its inhibition through rosiglitazone treatment (63). Given that the phosphorylation of JNK is increased in various insulin resistant models as previously mentioned, it is likely that this protein kinase is involved in mediating insulin resistance.

1.4e Protein Kinase C (PKC) and Insulin Resistance in Skeletal Muscle

The PKC's are part of a large family of protein serine/threonine kinases consisting of ~10 isoforms that are divided into 3 subfamilies. Together, PKC's regulate a wide range of cellular processes, such as cell growth and differentiation, and participate in several signalling cascades (65).

The different PKC isoforms have been divided into 3 subfamilies based on the substrates required for their activation (65). These groups are: the conventional calcium and diacylglycerol-dependent PKC's (cPKC, α, βI-II, and γ), novel calcium-independent PKC's (nPKC, δ, ε, η, and θ), and atypical calcium and diacylglycerol-independent PKC's (aPKC, ζ and ι/λ) (66).

Studies in L6 skeletal muscle cells have shown that high levels of the saturated FFA palmitate induced insulin resistance, which was correlated with a 5-fold increase in PKCζ activity (35). However, in the presence of Ro 31.8220, a PKC inhibitor the palmitate-induced insulin resistance was attenuated (35). Furthermore, PKCθ has been reported to phosphorylate IRS-1 on ser^{1101}, preventing downstream insulin signalling in C2C12 skeletal muscle cells and 3T3-L1 adipocytes (43). In addition, studies have shown that there is a link between FFA-induced insulin resistance and increased PCK activity *in vivo* (66). Lipid infusions in rats and humans impaired insulin-stimulated glucose disposal in skeletal muscle, as well as promoted increased activation of PKCθ and PKCδ (67). Taken together, FFA potently increases PKC activity, which may mediate insulin resistance in skeletal muscle and fat tissue.

1.4f IKK/ NFκβ Pathway and Insulin Resistance in Skeletal Muscle:

NFκβ is a family of transcription factors that regulate the expression of many genes including pro-inflammatory genes. The NFκβ proteins consist of five members, which include p65, p50, RelB, and c-Rel. Dimerization of two NFκβ family members is necessary for it to bind to DNA, and induce transcriptional modification in the cell. In skeletal muscle, the predominant activating NFκβ dimer is the p50-p65 heterodimer (44). NFκβ is predominantly found in the cytoplasm, where it is associated with its respective inhibitory protein inhibitor of kappa β (IKβ). Numerous stimulants, including cytokines, reactive oxygen species (ROS), hyperglycemia, and FFA, can activate IKβ kinase (IKK), which is upstream of IKβ. Once IKK becomes activated it causes rapid IKβ phosphorylation, which leads to IKB dissociation from NFκβ, and IKβ polyubiquitination and degradation by proteosomes located in the cytoplasm (44). This event allows NFκβ to be liberated from IKβ inhibition, which is now free to enter the nucleus and stimulate transcription of genes, as seen in figure 6.

The IKβ gene family mediating NFκβ inhibition includes seven members IKβα, IKBβ, IKBε, IKBγ, Bcl-3, the precursor Rel proteins p100, and p105. Of these members IKBα and IKBβ are the subunits of IKβ that interact with NFκβ and inhibit its DNA-binding (44). IKBγ acts as the regulatory subunit in the IKβ/ NFκβ inhibitor complex.

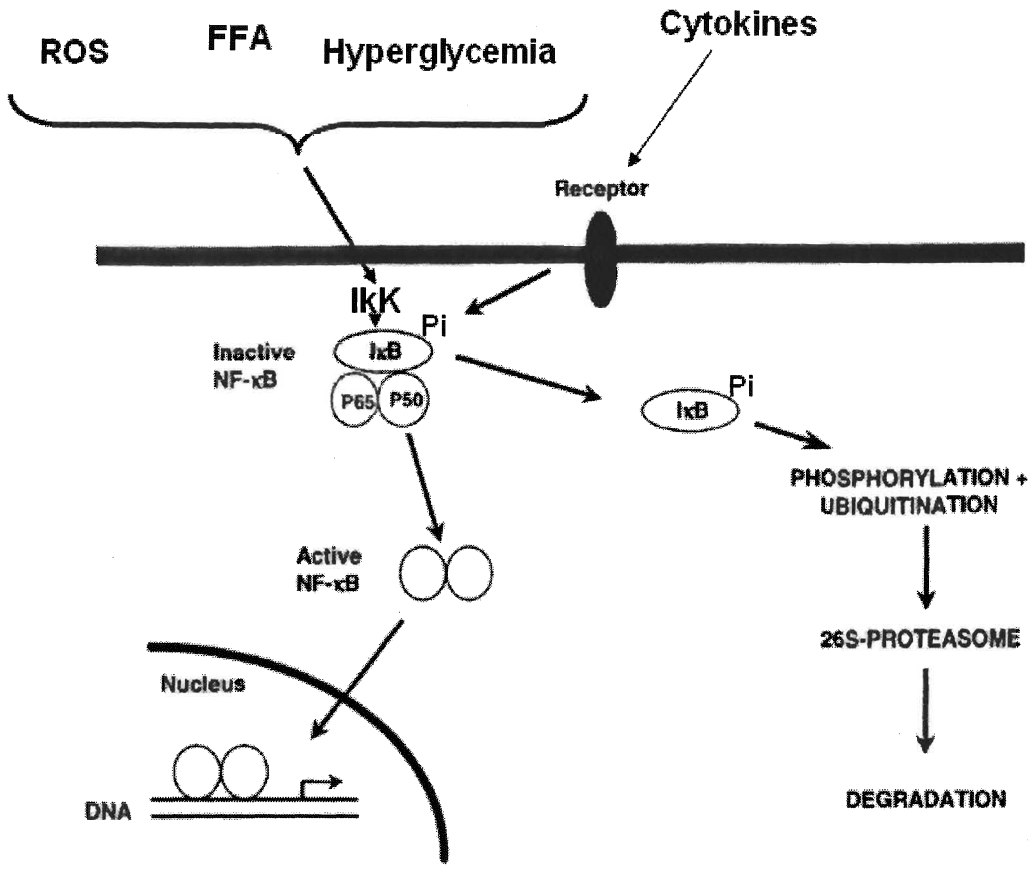


Figure 6: The various stimuli that regulate the NFκβ pathway

Studies in L6 skeletal muscle cells have shown that palmitate-induced insulin resistance is mediated by NFκβ (34). In the presence of SN50, a peptide inhibitor of NFκβ, the decrease in insulin-stimulated glucose uptake in L6 myotubes was attenuated (34). In addition, evidence collected from individuals with diabetes have suggested that the IKK/NFκβ inflammatory pathway is altered in patients with type 2 diabetes, and maybe responsible for the molecular mechanism causing skeletal muscle insulin resistance (44). Studies have shown that vastus lateralis muscle samples from patients with diabetes have a 60% decrease in IKBβ protein expression, when compared to matched control subjects, indicating increased NFκβ activity (44). Furthermore, increased

activation of IKK is linked to increased TNFα expression in skeletal muscle and adipose tissue (44). TNFα causes decreased glucose uptake and GLUT4 translocation to the plasma membrane in primary rat muscle cells, by impairing insulin stimulation of the insulin receptor and IRS-1/PI3-K association (68).

Exercise training increases insulin sensitivity and some of the benefits of exercise may be due to its effects on the NFκβ pathway. Exercise training in patients with diabetes resulted in a 50% increase in IKBα and IKBβ protein, and a 40% decrease in TNFα production (44). Additionally, exercise training has also been shown to inhibit NFκβ activation (64) . These finding indicate that the IKK/NFκβ pathway plays a role in mediating insulin resistance, and the benefits of physical activity in individuals with diabetes is due to the modulation of this pathway.

There are also reports that administration of an inhibitor of IKK, aspirin (salicylate) in patients with type 2 diabetes resulted in significant reduction of blood glucose levels, indicating that IKK is a key player in mediating insulin resistance (69). As such, future drug therapies that target IKK inhibition and the NFκβ pathway may provide significant improvements for regulating blood glucose levels in subjects with diabetes.

1.4g mTOR and p70 S6K Pathway and Insulin Resistance in Skeletal Muscle

The mammalian target of rapamycin (mTOR), also known as RAFT1 or FRAP is a 289 kDa serine/threonine kinase whose activation is increased by both nutritional (amino acids) and hormonal (insulin) factors (70). This kinase also plays a key role in cell growth and nutritional homeostasis, and may be abnormally regulated in tumors (71). Furthermore, mTOR is the mammalian counterpart of *Saccharomyces cerevisiae* TOR1 and TOR2, which is a member of the PI3-K-related family of proteins, that contains a C-

terminal lipid kinase domain and is a downstream effector of Akt (70;72). mTOR exists as two different complexes, a rapamycin-sensitive complex, with the regulatory-associated protein of mTOR (raptor) (TORC1), and a rapamycin-resistant complex with rapamycin-insensitive companion of mTOR (rictor) (TORC2) (73).

The signalling between Akt and mTOR involves the tuberous sclerosis complex (TSC), which consists of TSC1 and TSC2, and Rheb-GTP (a member of the Ras family) (70). Rheb has intrinsic GTPase activity and is able bind to both GDP and GTP. Both conformations of Rheb may directly bind to mTOR to modulate its activity (74). In the GTP-bound form Rheb increases mTOR activity. This process is mediated when Akt becomes phosphorylated, which will subsequently inactivate TSC and drive the intrinsic GTPase activity of Rheb towards the GTP-bound conformation (74). Conversely, in the GDP-bound form Rheb represses mTOR activity (70;74). This is achieved by increased TSC activity through the phosphorylation of AMPK, which drives the intrinsic GTPase activity of Rheb towards the GDP-bound conformation (75).

mTOR may be phosphorylated on different serine residues to increase its activity. For example, the PI3-K/Akt signaling pathway phosphorylates mTOR on the Ser^{2448} residue, which enhances its activity. Similarly, mTOR may become autophosphorylated at the Ser^{2481} residue, leading to its activation (71). Different subunits of TORC1, such as PRAS40 (praline-rich Akt substrate 40) are required for full mTOR activation (74). Recent studies by Vander Haar et al, 2007 has demonstrated that activation of mTOR through Akt is dependent on proline rich Akt substrate (PRAS40) (76). Inhibition of mTOR is dependent on the interaction of PRAS40 with mTOR on its kinase domain. This was observed in modified 293T cells that had a constitutively active mutant of mTOR

that was suppressed in the presence of PRAS40 (76). Conversely, mTOR is negatively regulated by AMPK, through at least two independent mechanisms (74). AMPK may phosphorylate TSC2 on multiple residues, which increases TSC2 activation (75). Secondly, AMPK can directly phosphorylate mTOR to decrease its kinase activity (75).

mTOR has a downstream effector known as p70 S6K, which is a mitogen activated, 70 kDa serine/threonine kinase that plays a role in regulating the translation of a class of mRNA transcripts which contain an oligopyrimidine tract at their transcriptional start sequence (77). This class of mRNA transcripts encodes several of the mechanisms for the protein synthetic apparatus, and may represent up to 20% of all cellular mRNA (77). This is accomplished when activated p70 S6K phosphorylates the 40S ribosomal subunit protein S6, thereby promoting the translation of mRNAs (47). p70 S6K is believed to be regulated through mTOR activation. The TORC1 complex induces phosphorylation of p70 S6K and the translation inhibitor factor eukaryotic translation initiation factor 4E binding protein (4EBP1) (73). In addition, p70 S6K is also negatively regulated by AMPK, which inhibit its activation by preventing phosphorylation of its upstream activator mTOR (78).

Activation of the mTOR and p70 S6K pathway by increased amino acid levels has been shown to desensitize insulin's action in L6 myotubes and 3T3-L1 adipocytes through a mechanism that involves increased serine phosphorylation of IRS-1 (79). Indeed, the activity of mTOR and p70 S6K are increased in the liver and skeletal muscle of rats that are fed a high fat diet, indicating that this signalling pathway may play a role in inducing insulin resistance *in vivo* (56). *In vitro* the effect of mTOR-induced insulin resistance is largely reversed using the mTOR inhibitor rapamycin. Rapamycin

specifically inhibits mTOR by interacting with the FKBP-rapamycin binding pocket of TOR, adjacent to the catalytic site of the kinase (72). Studies using insulin resistant 3T3-L1 adipocytes exhibited enhanced insulin-stimulated 2-[^{3}H]deoxy-D-glucose uptake when pretreated with rapamycin, suggesting that mTOR plays a role in down regulating the insulin signaling cascade (80). Also, rapamycin administration in mice decreased serine phosphorylation of IRS-1 and prolonged insulin-stimulated PI3-K activity in skeletal muscle (57).

Recently Mordier et al, 2007 showed that the FFA palmitate increased the phosphorylation of mTOR in rat hepatocytes (45). Palmitate-treated cells exhibited a rightward shift of the insulin dose-response curve for Akt phosphorylation, indicating insulin resistance. Additionally, hepatocytes incubated with palmitate (0.4mM) for 22h displayed hyperphosphorylation of IRS-1 at serine residues 632/639, thereby inhibiting downstream insulin signalling (45). Other *in vitro* studies in skeletal muscle cells and adipocytes demonstrate that amino acids induced phosphorylation of mTOR and reduced insulin-stimulated tyrosine phosphorylation of IRS-1 and decreased the activity of PI3-K (81).

Directly downstream from mTOR, p70 S6K (S6K1) has also recently been implicated in inhibiting normal insulin signaling in skeletal muscle and adipose tissue (72). *In vitro* studies have shown that treatment with S6K1 siRNA significantly increased insulin-stimulated Akt phosphorylation and increased insulin sensitivity in MEF mouse embryonic fibroblast cells (82). In addition, knockout mice that have a S6K1 deletion exhibit decreased IRS-1 phosphorylation on ser^{307} and ser$^{636/639}$ when subjected to a high fat diet, compared to their wild-type counterparts (56). Thomas et al, 2004 has also

demonstrated that S6K1-deficient mice are protected from obesity when subjected to a high fat fed diet (47). Conversely, elevated S6K1 has been correlated with decreased Akt activity in obese insulin-resistant rodents (47). Further insight into the molecular mechanism linking S6K1 to serine phosphorylation of IRS-1 was seen by studies involving the disruption of the TSC1-TSC2 tumor suppressor complex (57). Inhibition of this complex in mouse embryonic fibroblasts causes ser^{1101} phosphorylation of IRS-1 and degradation, as well as inhibition of IRS-1 transcription (57). Moreover, S6K1 was found to be constitutively active in TSC1-TSC2-deficient skeletal muscle cells (57).

The mTOR and p70 S6K pathway regulates insulin-stimulated glucose uptake through a negative feedback mechanism. Once blood glucose homeostasis is achieved, mTOR and p70 S6K inhibits the insulin signal by ser^{307} and $ser^{636/639}$ phosphorylation of IRS-1. However, chronic activation of the mTOR and p70 S6K pathway through various stimuli (as mentioned earlier) may cause insulin resistance in skeletal muscle, liver, and adipose tissue.

1.4h Free Fatty Acids (FFA) Induce Insulin Resistance in Skeletal Muscle

Non-esterified fatty acids (NEFA) play an important role in the establishment of insulin resistance and type 2 diabetes (49). Studies using NMR spectroscopy demonstrated a strong relationship between the accumulation of intramyocellular triglyceride content and skeletal muscle insulin resistance (83;84). This relationship is believed to be explained by the Randle hypothesis, whereby FFA compete with glucose for substrate oxidation and that elevated fat oxidation mediates the insulin resistance associated with obesity (85). Randle proposed that increased FFA concentration leads to increased mitochondrial acetylCoA/CoA and $NADH/NAD^+$ ratios, which subsequently

inhibit pyruvate dehydrogenase activity and cause increased citrate levels that inhibits phosphofructokinase activity (85).

In addition, it has been reported that increased FFA exposure in skeletal muscle cells inhibits downstream insulin-stimulated Akt phosphorylation. This inhibition has been linked to an increase in ceramide concentration from fatty acyl-CoA metabolism (49). The increase in ceramide may then in turn, activate a series of phosphatases such as phoshatase 2A that will dephosphorylate Akt. Thus Akt remains inactive in the presence of FFA, diminishing the insulin-stimulated glucose uptake response in skeletal muscle.

Furthermore, studies have examined the effect of saturated fatty acids on glucose uptake in skeletal muscle cells (86). Dimopoulos et al, 2006 has shown that L6 skeletal muscle cells that were incubated with palmitate at a concentration of 0.75mM for 16 hours, demonstrated a significant decrease in GLUT4 translocation to the plasma membrane (86). This reduction in GLUT4 translocation was correlated to the decrease in glucose uptake observed in these muscle cells.

Additionally, increasing FFA levels in rats by an intralipid infusion resulted in an twofold increase in ser^{307} phosphorylation of IRS-1 and a decrease in insulin sensitivity (87). Elevated FFA in overweight individuals may explain their greater risk of developing insulin resistance and type 2 diabetes (36). Insulin resistance is associated with the accumulation of intramyocellular lipids, which in itself do not appear to cause insulin resistance. However, they are markers for other lipid intermediates such as fatty acyl-CoA, ceramides or diacylglycerols, which can directly alter the insulin signalling cascade (36). A summary of the various kinases implicated in mediating insulin resistance through serine phosphorylation of IRS-1 is shown in figure 7.

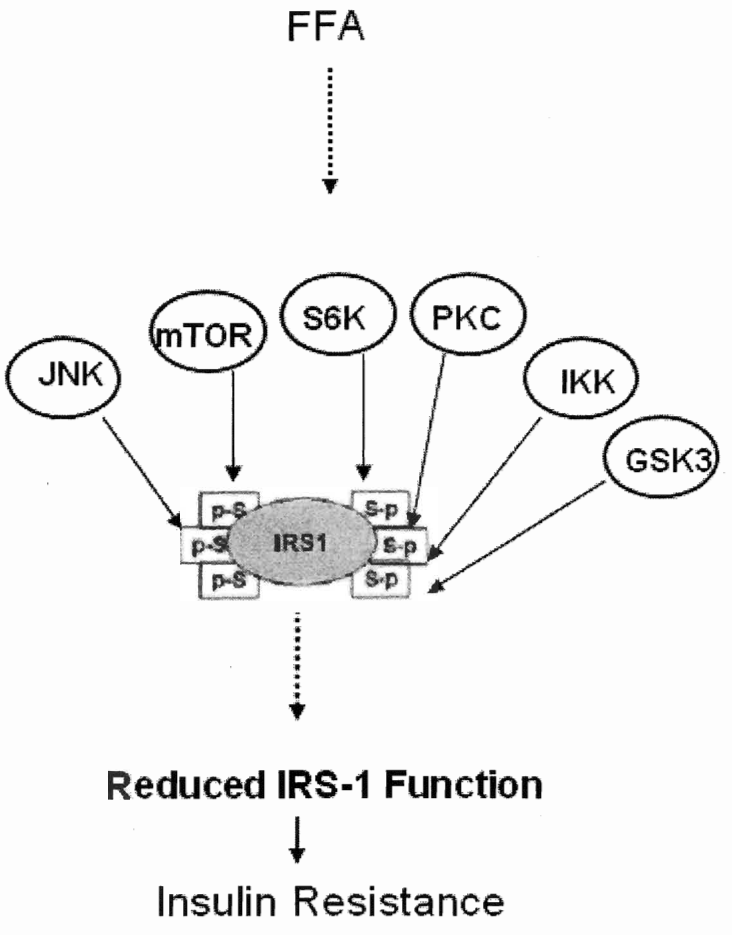


Figure 7: Serine phosphorylation of IRS-1 and potential stimuli involved in regulating the activity of IRS-1

1.5 AMP-activated Protein Kinase Pathway

AMPK is a 62 kDa serine/threonine kinase that plays a key role in the regulation of energy balance in the cell. AMPK is activated in response to an increase in the AMP/ATP ratio. A reduction in ATP levels as a function of increased muscle activity leads to activation of AMPK, which phosphorylates and inhibits acetyl-CoA carboxylase (ACC). This inhibition causes a decrease in the levels of malonyl-CoA, which itself is a potent inhibitor of carnitine palmitoyltransferase I (CPT I) (88). The loss of CPT 1

inhibition will promote enhanced beta-oxidation of fatty acid in muscle mitochondria, which in turn replenishes ATP levels (89).

Structurally, AMPK exists as a heterotrimer containing a catalytic α subunit and regulatory β and γ subunits (90). Presently, homologues of each AMPK subunit have been identified in every eukaryotic species, ranging from fruit-flies, worms, and yeast, to fish and mammals (91). AMPK is also related to a protein kinase complex in *Saccharomyces cerevisiae* termed SNF1, and shares similar structure and function (91). In mammals the following isoforms of the different AMPK subunits exist α1, α2, β1, β2, γ1, γ2, and γ3 (89).

The N-terminal section of the α subunit of AMPK contains the serine/threonine kinase catalytic domain. In addition, this subunit of AMPK contains the thr^{172} residue, which becomes phosphorylated and is essential for AMPK activation. On the other hand, the C-terminal segment of the α subunit of AMPK is required for association with the β and γ subunits (92). The physiological function of both the β subunit and γ subunit of AMPK has not been widely studied. However it has been reported that the β subunit acts as a scaffold to link the α and γ subunits of AMPK together. The γ subunit has been shown to play a role in glucose metabolism and fat oxidation (93).

AMPK phosphorylation and activation is regulated by the upstream protein kinases LKB1 (91), calcium/calmodulin-dependent protein kinase (CaMKK), and TGFβ-activated kinase-1 (TAK1), and by SIRT1 (94). LKB1 is a tumor suppressor that phosphorylates the thr^{172} residue of AMPK to promote its activation (91). Studies have demonstrated that AMPK activation is abolished in human fibroblast cells lacking expression of LKB1, or following inhibition of LKB1 (95;96). Similarly, phosphorylation

of AMPK is prevented in L6 skeletal muscle cells in the presence of STO-609, a CaMKK inhibitor (97). Furthermore, in mice embryos that expressed a TAK1-knockout, pharmacological activation of AMPK by AICAR (5-amino 4-imidazolecarboximide riboside) and metformin was completely abolished (98). Also, recent evidence has shown that in the presence of splitomicin, a SIRT1 inhibitor, activation of AMPK by the polyphenol resveratrol was inhibited (94).

There are many reports that AMPK acts to increase glucose uptake in skeletal muscle cells. Studies using L6 and C2C12 skeletal muscle cells *in vitro* have shown that AMPK activation by resveratrol increases glucose uptake. Conversely, inhibition of AMPK by the AMPK inhibitor compound C prevents resveratrol-stimulated glucose uptake (99;100). In addition, studies *in vivo* have demonstrated that transgenic animal that exhibited a deletion in AMPK α2 catalytic subunit experienced insulin resistance and decreased insulin secretion (101). Taken together, these funding suggest that AMPK is a key player in energy homeostasis and glucose uptake in skeletal muscle.

THE POLYPHENOL RESVERATROL

1.6a Sources of Resveratrol

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is a polyphenolic compound, classified under the stilbene class of polyphenols (102). Many stilbenes found in plants act as phytoalexins, which are compounds that are generated with the help of the enzyme stilbene synthase, and only in response to injury or infection (102). Stilbenoids are antibacterial and anti-fungal chemicals produced by plants as a defense against infection by invading pathogens.

Resveratrol is a widespread phytoalexin that can be found in at least 72 species of plants, spread over 31 genera and 12 families (103). It was first isolated in 1940 as a constituent of the white hellebore roots *Veratrum grandiflorum*. Presently, there are numerous dietary sources of resveratrol that range from raspberries, mulberries, plums, berries of Vaccinium species, including blueberries and cranberries, some pines, such as Scots pine and eastern white pine, and the roots and stalks of giant knotweed and Japanese knotweed, peanuts, cocao, and in the highest concentrations in red grape skin and red wine (103). On average, ordinary non-muscadine red wine contains between 0.2 and 5.8 mg of resveratrol per liter of wine, as seen in table 3 (104). Resveratrol content in white wine is much lower, due to the different ways that the wines are produced. Red wine is fermented with the skin still attached to the grape, which allows resveratrol absorption into wine. White wine is fermented after the skin has been removed from the grape, thereby lowering resveratrol content (105).

The benefits received from dietary polyphenol consumption are not limited to today's western society. In the 1980's Japanese scientists observed that the dried roots of *Polygonum cuspidatum* have been utilized in traditional Japanese and Chinese medicine for hundreds of years. The medicinal product known as "Kojo-Kon" was used to treat a wide variety of aliments ranging from fungal diseases, skin inflammations, heart disease, and blood vessel obstructions (102). Resveratrol was the primary active ingredient in this herbal remedy, with its properties being further supported by present studies using *in vitro* and *in vivo* animal models.

Table 3: Dietary sources of resveratrol (105)

Content of Resveratrol in Wines and Grape Juice		
Beverage	***Resveratrol Concentration (mg/L)***	***Total Resveratrol in 5oz Glass (m***
Muscadine Wine	14.1-14.4	2.22-2.6
Red Wine	1.92-12.59	0.29-1.89
Red Wine	1.98-7.13	0.30-1.07
Red Grape Juice	1.14-8.96	0.17-1.30
Rose Wines	0.43-3.52	0.06-0.53
Pinot Noir	0.40-2.0	0.06-0.30
White Wines	0.05-1.80	0.01-0.27
Content of Resveratrol in Food		
Food	***Serving (g)***	***Total Resveratrol (mg)***
Peanuts (raw)	146	0.01-0.26
Peanuts (boiled)	180	0.32-1.28
Peanut Butter	258	0.04-0.13
Red Grapes	160	0.24-1.25

1.6b Chemical Structure of Resveratrol

Although resveratrol is classified as a stilbene, it differs in the structure of classic stilbenes by containing three hydroxyl groups that participate in an extensive three dimensional hydrogen-bonding (H-bonds) network (106). These H-bonds may readily form and break H-bonds with other neighboring phenolic oxygen, thus explaining its antioxidant properties. The precursor for all stilbenes is phenylpropanoids, which include flavonoids, as well as resveratrol (106). The aromatic ring structure of resveratrol (figure 7) allows it to bind with many different enzymes and receptors (107).

Resveratrol exists as two different isomers which are labeled *cis*, and *trans* (107). The primary source of resveratrol found in nature and in industry is the *trans* isomer, which is also the most biologically active form. *Trans*-resveratrol becomes isomerised to the *cis* isomer under exposure to light as shown in figure 8 (107).

Cis- resveratrol is less biologically active that *trans*-resveratrol, demonstrating the difference in the molecular behavior of the two isomers, which is governed by their three-dimensional molecular structure (106). For instance, resveratrol interacts with the estrogen receptor in a stereoselective manner. Studies of the two resveratrol isomers (*cis*, and *trans*-resveratrol) on MCF-7 breast cancer cells have demonstrated that the *trans*-resveratrol isomer is a much stronger mixed estrogen receptor-α (ER-α) agonist/antagonist than the *cis*-resveratrol isomer (107). Recent studies using molecular dynamics and docking experiments have demonstrated that *trans*-resveratrol has greater hydrogen bond interactions binding to the ER-α than the *cis* isomer, providing an explanation for the stereoselective ligand binding with the protein.

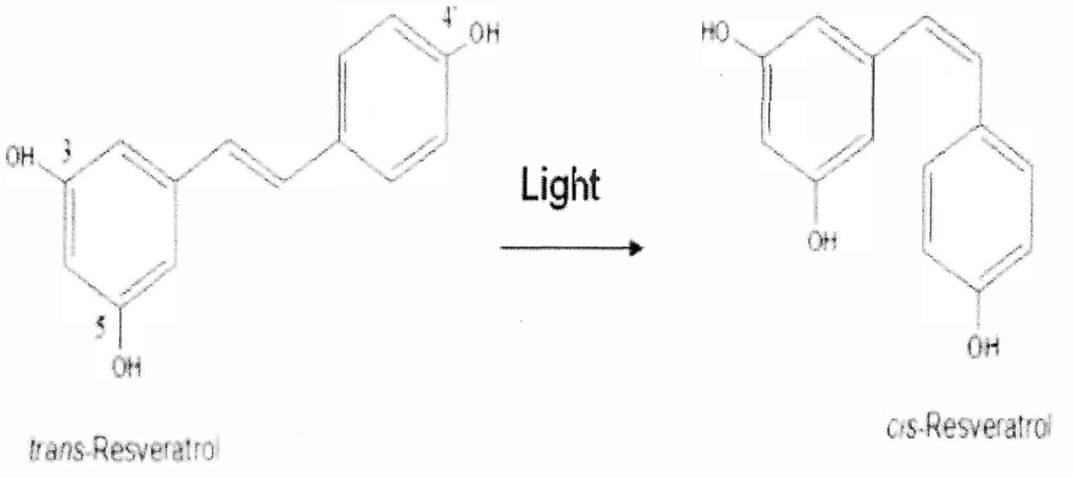


Figure 8: Resveratrols isomerization by exposure to light.

1.6c Bioavailability of Resveratrol

There are few studies that examined the bioavailability of resveratrol. The absorption of resveratrol in rats was first examined in 1996. Studies by Bertelli et al, used red wine with established resveratrol content (6.5mg/L) to examine the bioavailability of resveratrol in male Wistar rats that were fed 4mL of red wine per day (equal to 86μg/Kg of resveratrol), for 15 days (108). The results demonstrated that the resveratrol contained in wine was quickly absorbed, reaching its maximal blood concentration after 60 minutes of wine ingestion. After a very short period of time, resveratrol was detected in the liver and kidneys of rats. The kidney showed signs of decreased resveratrol concentration after 1 hour, indicating that it may be the preferential organ of excretion (108).

Studies by Marier et al, in 2002 used a single oral dose of resveratrol in rats and examined its metabolism using electrospray ionization-liquid chromatography (108). Rats that were administered an oral dose of resveratrol (50 mg/kg body weight) experienced a rapid drop in serum levels of resveratrol within 8 minutes. However, detectable levels of resveratrol remained for 12 hours, due to enterohepatic recirculation from the liver (108). This study demonstrated that the bioavailablity of resveratrol undergoes extensive first-pass glucuronation. This study also concluded that the terminal elimination half life of resveratrol is approximately 1.6 hours in rats (108).

It wasn't until 2003 that the metabolism and bioavailability of resveratrol was studied in humans. Subjects were administered 10mg of resveratrol per 70Kg of body weight. Serum levels of resveratrol peaked at 1.82μM within 30 minuets of its ingestion, and took between 7-10 hours to excrete most of it in the urine (102). Although the resveratrol concentration being administered is fairly high in human studies, the

absorption of it is fairly low, with approximately 75% of it being excreted (102). The oral bioavailability of resveratrol is negligible due to rapid metabolism and the consequent formation of various metabolites as resveratrol glucuronides and sulfates (102). It was shown that the liver and kidney are the main targets of resveratrol uptake, as well as being key players in the absorption and bioavailability of many other dietary polyphenols (102).

To increase the bioavailability of resveratrol, a study by Hung et al, 2008 demonstrated that resveratrol transported via the skin had lower metabolism and a prolonged half-life compared to oral administration (109). In addition, applying resveratrol to the skin also provides protection against UV exposure by the sun (109). Currently, there are several phase I clinical trials underway for oral administration of resveratrol in humans (109;110). Some studies are providing an oral dose of resveratrol as high as 7.5g per day (109).

1.6d Toxicity of Resveratrol

In vitro, resveratrol is used in fairly high concentrations ranging from 1-100μM in various cells including vascular, cancer, and skeletal muscle (99). However, high concentrations of resveratrol over 100μM have been reported to have severe toxicity in C2C12 skeletal muscle cells (100).

There are a few *in vivo* studies that have examined the toxicity of resveratrol in animals. A study by Juan et al, 2002 administered resveratrol (20mg/Kg body weight) orally to rats for 4 weeks and reported no toxic effects (111). A single highly concentrated oral dose of resveratrol (2000mg/Kg body weight) did not have any detectable toxic effect in rats as well (111). However, it has been demonstrated that there

are toxic effects in rats administered 300-3000mg of resveratrol by gavage for 4 weeks (104). Clinical signs of toxicity included decreased body weight, reduced hemoglobin, increased white blood cell number, and increased kidney weight with associated nephropathy was observed in rats receiving 1000mg of resveratrol or more over the course of 4 weeks (104). In human subjects, resveratrol has a short half-life and there is no established toxic dose (109).

1.6e Transport of Resveratrol through the Cell

Resveratrol has low water solubility and thus, must be bound to proteins and/or conjugated to remain in high concentrations in serum (112). A study by Janin et al, 2004 demonstrated that resveratrol interacts with lipoproteins of rat liver cells *in vivo*. Specifically, resveratrol was found to associate with albumin, which is well known to bind and carry a variety of other amphiphilic molecules (112). For example, it was shown that querectin, another polyphenolic compound, interacts with very high affinity with albumin in rat plasma and human serum albumin (113). In addition, a recent study by Walle et al, 2004 observed that only trace amounts (<5 ng/mL) of unconjugated resveratrol was found in the plasma after administration of 25mg/ ^{14}C-resveratrol to six healthy human subjects (114).

In vitro assays also demonstrated that resveratrol added to plasma increased with increased lipoprotein content (115). The concentration of resveratrol in plasma increased with the order of the lipid content, i.e. HDL < LDL < VLDL, and that resveratrol is more aggregated with lipoproteins than with lipoprotein-free proteins. This binding occurs *in vivo* as well, since it is found in the presence of LDL isolated from blood samples of healthy individuals (115).

Studies using HepG2 cells *in vitro* have examined the kinetics of labeled resveratrol transport (116). The uptake of resveratrol in HepG2 cells involves both passive diffusion and a carrier-mediated process. Similar studies have examined the transport of resveratrol through human intestinal epithelial cell (Caco-2) (117). Kaldas et al, 2003 demonstrated that resveratrol passively crosses the plasma membrane of Caco-2 cells at a rapid, dose-dependent rate. Taken together, these studies suggest that resveratrol is quickly absorbed through a mechanism that may be passive or carrier-mediated.

1.6f Biological Effects of Resveratrol

Numerous *in vitro* and *in vivo* studies have demonstrated the different beneficial biological effects of resveratrol (99;118-120). Presently, it has been established that resveratrol possesses antioxidant (121), anticardiovascular diseases (120;122), anticancer (119), antiaging (118), and antidiabetic properties (99;118;123). In the following sections all of the above mentioned biological effects of resveratrol are reviewed.

1.6g The Antioxidant Properties of Resveratrol

Reactive oxygen species (ROS) are small molecules with unpaired valence shell electron that are highly reactive in biological tissue (124). ROS are generated as a natural byproduct of oxygen metabolism and play a critical role in cell signalling (125). Excessive ROS levels also termed oxidative stress may be due to increased production or decreased removal of ionizing radiation and other environmental factors that may result in increased ROS levels. The main ROS are the hydroxyl radical (OH), superoxide (O_2^-), nitric oxide (NO^-), and hydrogen peroxide (H_2O_2) (124). These ROS may activate signalling pathways such as JNK, PI3-K/Akt, and NFκβ, which may lead to the modulation of genes and increased cell apoptosis (124).

It is suggested that the majority of intracellular ROS is derived from the mitochondria and is counterbalanced by an intricate antioxidant defense system (124) that include the enzymatic scavengers superoxide dismutase (SOD), and glutathione peroxidase that convert O_2^- into water (126). The body may also combat oxidative stress with antioxidants, compounds that stop oxidation of important cellular targets, and with free radical scavengers that act on free radicals to make them stable products. There are a variety of naturally occurring antioxidants found in fruits and vegetables, as well as synthetic ones created by the pharmaceutical industry (127). Figure 9 summarizes some of the events that may occur by ROS.

Resveratrol is established to have antioxidant properties (121). Flavonoids and other polyphenols like resveratrol can donate hydrogens or interact with free radicals like superoxide and hydroxide ions. By doing so, resveratrol converts these free radicals into harmless, unreactive compounds in the body (128). For example, studies by Olas et al, 2001 have shown that resveratrol decreased free radical formation in platelets (129). Platelets *in vitro* were stressed, and ROS such as superoxide and hydrogen peroxide were measured using chemiluminescence. Resveratrol was shown to inhibit chemiluminescence and generation of ROS in these blood platelets, as well as the production of thrombin and thiobarbituric acid-reactive substances (TBARS) (129). Resveratrol is able to counteract oxidative damage caused by H_2O_2 in rat primary cortical astrocytes *in vitro* (126).

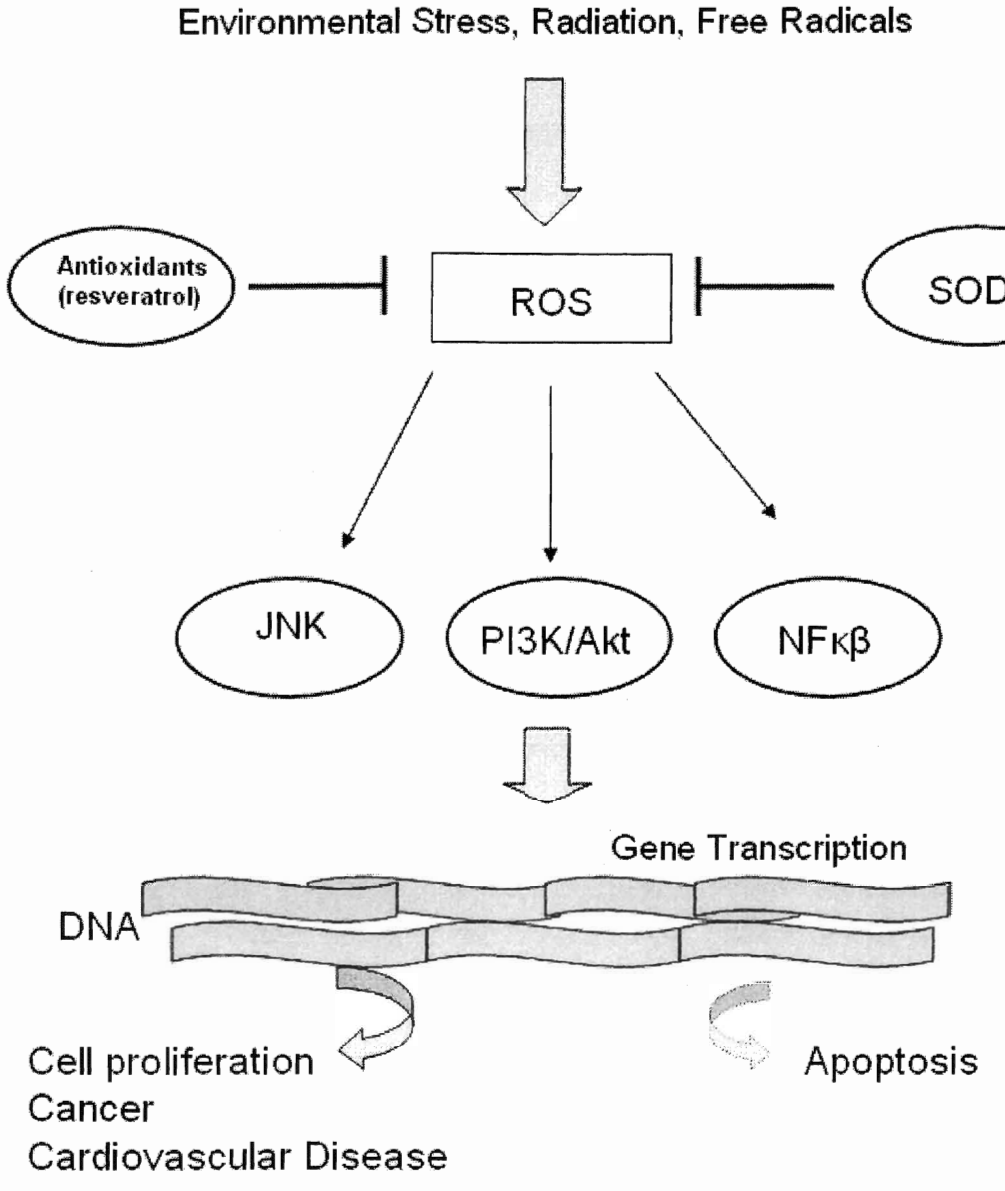


Figure 9: The major signalling pathways effected in response to ROS

Increased ROS levels lead to DNA damage and mutation. Studies in normal rat fibroblasts, mouse mammary epithelial cells, human breast, and human prostate cancer cells showed that resveratrol significantly reduces DNA fragmentation, as assessed by single cell gel electrophoresis (130). Resveratrol was shown to prevent increased ROS levels following exposure to oxidative agents like tobacco smoke (130).

In addition, resveratrol was shown to prevent oxidative stress in human lung fibroblasts by increasing mitochondrial superoxide dismutase (MnSOD) which, by itself is capable of reducing intracellular oxidative stress (131).

1.6h Anti-cardiovascular Disease Properties of Resveratrol

Interest in the study of polyphenolic compounds present in red wines has grown since epidemiological studies have observed a decrease in cardiovascular disease in individuals that frequently drink red wine (108). This observation was prominent in France, and also helped explain the "French paradox", which is the inverse relationship between the high consumption of fatty foods, and the low incidence of cardiovascular disease found in the French population (108). Resveratrol is able to inhibit the peroxidation of lipid membranes, to decrease the concentration of low (LDL) and very low (vLDL) density lipoproteins, and inhibit platelet aggregation (108). These three factors are all strongly linked to cardiovascular disease and stroke. Resveratrol may also decrease smooth muscle cell proliferation, and act as a vasorelaxing agent (132).

Furthermore, there has been research that implicates resveratrol in having cardioprotective action directly on the heart. Studies by Liew et al, 2005 have demonstrated that resveratrol exerts multiple direct actions on guinea-pig cardiac myocytes (133). Resveratrol significantly decreased the duration of action potentials, as well as peak L-type calcium (Ca^{2+}) currents. This suggests that acute treatment with resveratrol increases Ca^{2+} sensitivity in cardiac myocytes and thereby reduces cardiac stress (133).

Studies have also investigated the potential cardio protective effects of resveratrol on the diabetic heart. For example, Dekkers et al, 2007 used normal and diabetic rats that

were orally fed resveratrol (2.5mg/Kg/day) for 1 week to examine myocardial infarct size and cardiomyocyte apoptosis upon global ischemia in isolated rat hearts (134). They found that resveratrol decreased infarct size and apoptotic cell death in both normal and diabetic rat groups. The ability of resveratrol to mediate these cardio protective effects was attributed to the modulation of several stress proteins such as heat shock protein 27 (HSP 27) and αβ-crystallin (134). Similar results were found by Aubin et al, 2008 in normal female rats fed a high fat diet for 8 weeks (135). These rats become obese and diabetic by the end of the 8 week period. A significant increase in systolic and diastolic blood pressure, as well as decreased acetylcholine-dependent relaxation of the aortic ring was seen in rats fed a high fat diet. However, administration of resveratrol (20mg/Kg/day) for 8 weeks significantly prevented these high fat diet-induced effects (135). More recently, a study by Lekli et al, 2008 attempted to investigate if resveratrol could improve postischemic cardiac function, infarct size, and arrhythmias in Zucker obese rats (136). After 14 days of resveratrol treatment (5mg/Kg), postischemic cardiac function was improved and the incidence of ventricular fibrillation and infarct size was reduced by 83% (136). Furthermore, they discovered that resveratrol increased GLUT4 expression and decreased endothelin expression and cardiac apoptosis in ischemic-reperfused hearts in the presence or absence of glucose intake (136).

1.6i Anticancer Properties of Resveratrol

The link between resveratrol and cancer prevention has been widely studied in the cancer field. Tumorigenesis is classified into three major stages; cancer initiation, promotion, and progression (119). Resveratrol has been established to have chemopreventive cancer proprieties, and can inhibit all three stages of cancer cell

progression (119). Resveratrol has also been shown to suppress the proliferation of a wide variety of tumor cells, including lymphoid, myeloid, multiple myeloma, prostate, stomach, colon, pancreatic, thyroid, melanoma head and neck squamous cell carcinoma; ovarian carcinoma, and cervical carcinoma (119). The anticancer effects of resveratrol are due to cell-cycle arrest, increased expression of protooncogenes (such as p21, and p53), and down-regulation of cancer cell survival. Resveratrol is known to suppress several transcription factors including, NF-kappa β (NFκβ), AP-1, Egr-1, and to inhibit protein kinases involved in cell survival, such as Akt, JNK, MAPK, and PKC (119).

Studies using androgen-insensitive prostate cancer cells has shown that resveratrol downregulates many of the genes involved in prostate cancer cell survival (137). In addition, resveratrol upregulates the expression of TNF-related apoptosis inducing ligands (TRAIL), thereby arresting prostate cancer development. Also, the combination of resveratrol and TRAIL enhanced the mitochondrial dysfunctions during cell apoptosis (137).

In addition, resveratrol has the ability to induce s-phase arrest in several human cancer cell lines. Studies by Joe et al, 2002 has examined the effects of resveratrol on six different human cancer cell lines, MCF7, SW480, HCE7, Seg-1, Bic-1, and HL60 (138). In five of these cancer cell lines, resveratrol induced significant growth inhibition with an IC_{50} value of 70-150μM. Also, following treatment with 300μM resveratrol, most of the cell lines were arrested in the S phase of the cell cycle (138). Resveratrol treatment for 2 days demonstrated significant reduction in cyclin B1 expression levels in all cancer cell lines, indicating that the cancer cell is not progressing through the cell cycle. Another

study by Ginkel et al, 2008 observed that resveratrol has the ability to inhibit uveal melanoma tumor growth *in vitro* and *in vivo* (139).

Recently a study by Schlachterman et al, 2008 demonstrated that resveratrol, in combination with other grape polyphenols, decreased the size of primary breast cancer tumors in nude mice (140).Peak brest cancer inhibition was observed using in situ image analysis at 5mg/Kg of resveratrol. Similar results were reported by Garvin et al, 2006 where resveratrol significantly inhibited brest cancer tumor growth and increased cancer cell apoptosis in nude mice (141).

1.6j The Prosurvival and Anti-Aging Properties of Resveratrol

Initial studies showed that caloric restriction in yeast resulted in cell survival and increased lifespan by increased activity of sir2 (142). Moreover, resveratrol is a potent activator of sir2 and its mammalian counterpart SIRT1 (142). Enhanced activation of Sir2 by resveratrol in the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* has also been linked to lifespan extension through a dietary restriction-like mechanism (143). Recently, it has been established that resveratrol has the ability to prolong the survival of mice on a high fat diet (118;144).

However, the mechanism of action of resveratrol to increase lifespan through Sir2 activity has been variable. Studies have also reported that resveratrol may not extend the lifespan of nematodes or fruit flies, or that it may do so through the interaction with other proteins that have yeast homologs, such as AMPK and protein kinase C (145;146). In addition, increased lifespan does not imply enhanced Sir2 activity, as several single-gene mutations are know to extend the lifespan of yeast in a Sir2-independent manner (146). Furthermore, *in vitro* assay has shown that resveratrol activates Sir2 to deacetylate a

fluorescent peptide substrate, it does not activate either Sir2 or the human ortholog SIRT1 towards more native peptide substrates (147). More recently, a study by Bass et al, 2007 has reported that their result on lifespan extension using resveratrol treatments on *C elegans* and *D melanogaster* has been inconsistent (145).

Taken together, resveratrol has been shown to have prosurvival proprieties through the activation of sirtuins. However, there are some reports that indicate resveratrol may prolong lifespan through different mechanisms.

1.6k The Sirtuin Family of Histone Deacetylases and Anti-Aging

Histone deacetylases (HDAC) are a class of enzymes that play a role in removing acetyl groups from an N-acetyl lysine amino acid or histones (148). Activation of HDAC causes hypoacetylation of histones, resulting in a stronger association between the nucleosome and the DNA wrapped around it (148). Thus, the tighter the association between the DNA and nucleosome the less chance transcription factors can access the DNA, thereby causing transcriptional repression (149).

The family of HDACs consists of 3 subclasses; RPD3 (class 1), HDA1 (class II), and sir2 (class III) (150). Class I and II of HDACs use zinc as cofactors to deacetylate substrates and are inhibited by trichostatin (TSA) (151). The class III of HDACs (group III) differs in that; they require nicotinamide adenine dinucleotide (NAD+) as a cofactor to deacetylate substrates and are inhibited by nicotinamide, splitomicin, and sirtinol (150;151). In addition, HDAC's are characterized by a domain of approximately 260 amino acids that have a high degree of structural similarity (152).

The sirtuins are class III histone and protein deacetylases (HDACs) that were named after their homology to the *Saccharomyces cervisiae* gene silent information

regulator 2 (Sir2) (150). There are seven mammalian homologs of sirtuins (SIRT1-7) (151). In yeast, Sir2 has been reported to mediate global metabolic changes and life span extension (153). The mammalian homologs of Sir2 have been shown to regulate many important biological process ranging from life span extension, to apoptosis, energy metabolism, muscle differentiation, and gluconeogenesis (150). In the following sections a review of the localization and function of the sirtuin family will be presented.

Sir2 involvement in life span extension: Initially it was demonstrated that caloric restriction (CR) can significantly increase the life span of *Saccharomyces cerevisiae* (yeast) by causing metabolic changes (154). The identification of sir2 as a potential molecular mediator of CR is a breakthrough in understanding the molecular mechanism underlying the process of aging. sir2 in yeast is a nuclear protein that can deacetylate histones, bind to telomeres, and induce transcriptional repression of the silent mating type loci; such as homothallic mating-type loci left (HML) to promote cell longevity. The aging of yeast can be mediated by the accumulation of extrachromosomal rDNA circles (ERCs) in the nucleus, which sir2 can inhibit (153). Studies that used deletions of sir2 in yeast demonstrate a dramatic increase in ERC formation, while shorting life span by approximately 50% (153). Conversely, studies that use overexpression of sir2 in yeast show decreased ERC expression, decreased histone acetylation, and an increase in life span by 30-40% (153).

In *Caenorhabditis elegans*, the yeast sir2p ortholog, sir2.1, extends life span via the forkhead (FOXO) transcription factor Daf-16 signalling pathway (150). This pathway is responsible for the early development states of growth arrest that is induced upon food limitation in nematode larva. Studies that duplicated the chromosomal regions containing

sir2.1 in the nematode extended its life span by up to 50% (155). Conversely, nematodes containing a mutated Daf-16 gene displayed a significant decrease in life span compared to wildtype nematodes.

In *Drosophila melanogaster*, the sir2 ortholog, dsir2 regulates life span extension under CR conditions (150). The ability of dsir2 to mediate life span extension is believed to be through the same pathway as Rpd3 histone deacetylase, given that Rpd3 is thought to negatively regulate dsir2, and CR induces a decrease in Rpd3 expression (150). Mutations in dsir2 have been shown to inhibit the CR-mediated increase in life span seen in flies (156). Furthermore, long-lived Rpd3 mutant flies demonstrate increased dsir2 expression levels that correlate with increase longevity (156).

The mammalian SIRT1-7: The mammalian NAD+ pathway is different from the yeast NAD^+ pathway in that, nicotinamide adenine mononucleotide (NAM) is directly converted to nicotinamide mononucleotide (NMN) by the enzyme nicotinamide phosphoribosyltransferase (NamPT) (150). The functions of each of the NAD^+-dependent sirtuins (1-7) may vary in a tissue-specific manner. SIRT1 is the most widely studied of all the mammalian sirtuins.

SIRT1: SIRT1 is a 120 kDa HDAC that is localized in the nucleus and cytoplasm (154). There have been an increasing number of studies focusing on the beneficial effects of SIRT1 activity in the amelioration of metabolic diseases such as type 2 diabetes (144). For example, studies have demonstrated that SIRT1 can augment insulin secretion in response to glucose in the β-cells of the pancreas (157). Moynihan et al, 2005 showed that beta cell-specific SIRT1-overexpressing (BESTO) transgenic mice had decreased expression of uncoupling protein 2 (UCP-2), which impedes H+ leakage to allow more

efficient coupling of electron transport (157). This in turn provides improved glucose tolerance and enhanced insulin secretion in the pancreatic β-cells.

Resveratrol has been used in numerous studies investigating the role of SIRT1 activation in mammalian tissues. Several studies implicate resveratrol in improving health and extend maximum lifespan of vertebrate fish by 59% in a SIRT1-dependent fashion (118;158). More recently, resveratrol was proposed to extend the lifespan of mice by 20% via SIRT1 activation (118).

A possible mechanism in which resveratrol interact with SIRT1 has been shown using a covalently attached flurophore, and 7-amino-4-methylcoumarin (p53-AMC) peptide (147). Resveratrol is able to bind to SIRT1 and causes a conformational change that better accommodates the attachment of SIRT1-substrates, such as p53 (147). SIRT1 has several residues such as Gln^{102}, Met^{104}, Phe^{105}, and Arg^{254} that likely interact with resveratrol once it is bound (147). The current hypothesis in resveratrol-mediated SIRT1 activation is via a single allosteric site on the SIRT1-substrate complex, to which structurally diverse compounds can bind (144). This theory is supported by Milne et al, 2007 who demonstrated that structurally diverse small molecule activators, unrelated to resveratrol activated SIRT1 up to 1000-fold more potent than resveratrol alone (144). Furthermore, all of the SIRT1 activators share the same allosteric compound binding site on amino acids 240-664, as well as the amino acids 183-225 N-terminal to the core SIRT1 domain (144).

SIRT1 has been reported to deacetylate many different molecules/substrates. For example, in the liver SIRT1 forms a substrate-complex with hepatocyte nuclear factor-4 (HNF-4) to deacetylate and activate peroxisome proliferator-activated receptor gamma

(PPARγ) coactivator-1α (PGC-1α) to promote gluconeogenesis following fasting (159). The combined role of SIRT1 and PGC-1α moves beyond the liver, to mitochondrial function and global energy metabolism as well. SIRT1-activated PGC-1α improves mitochondrial function in skeletal muscle and brown adipose tissue, leading to increased energy expenditure, enhanced exercise performance, and protection from diet-induced insulin resistance (123;150). In addition, SIRT1 has been shown to form a complex with FOXO1 and the promyelocytic leukemia protein PML to activate two insulin transcription factors, NeuroD and MafA, which may protect the β-cell from oxidative damage (150).

Recently, it was demonstrated that SIRT1 is an upstream activator of AMPK in hepatocytes (94). SIRT1 activation by resveratrol increased the phosphorylation of LKB1 and AMPK. Conversely, AMPK phosphorylation by resveratrol was abolished by pharmacological and genetic inhibition of SIRT1, suggesting that the ability of resveratrol to stimulate AMPK is dependent on sirtuins (94). In addition, NFκβ is a substrate for SIRT1 (160). Resveratrol-mediated activation of SIRT1 is able to directly interact with the RelA/p65 subunit of NFκβ to inhibit its transcription. SIRT1 deacetylates RelA/p65 on lysine 310 to prevent NFκβ-induced transcription of pro-inflammatory genes (160). Also, p53 acts as a substrate for SIRT1 in several cancer cell lines (161). Deacetylation of p53 is believed to explain some of the anti-cancer properties of SIRT1 (161).

SIRT2: Emerging research is beginning to focus on the function of other SIRT homologs (SIRT2-7) in mammalian tissue. SIRT2 is a tubulin-deacetylase protein that acts as a mitotic checkpoint to prevent chromosomal instability (162). SIRT2 may delay

cell cycle progression and control mitotic exit in cell cycle instabilities. Studies has shown that SIRT2 plays an important role in the pathology of brain tumor gliomas (162). In fact, SIRT2 is often downregulated or deleted in human gliomas, thus increasing their malignant progression (163). In addition, it was recently shown that SIRT2 may also play a role in myelinogenesis, as it was demonstrated to be localized to the outer loops of the myelin sheath in humans (150).

SIRT3: The SIRT3 protein deacetylase is localized in mitochondria and functions to regulate thermogenesis in mice (164). Upon exposure to cold temperatures, the expression of SIRT3 is upregulated in both white and brown adipose tissue. In addition, SIRT3 also activates mitochondrial genes such as PGC-1α and UPC-1 to modulate core body temperature (164).

SIRT4: This sirtuin homolog has been shown to be expressed in mitochondria and inhibit mitochondrial glutamate dehydrogenase (GDH) (165). Interestingly, SIRT4 appears to work in the opposite direction of SIRT1 to regulate insulin secretion by inhibiting GDH activity to prevent insulin secretion in mouse β-cells, in response to amino acids (165). Furthermore, the expression of SIRT4 is decreased in response to CR in β-cells, which is the reverse of SIRT1 expression during CR (165).

SIRT 5: The expression of SIRT5 is found only in mitochondria; however the function of SIRT5 is currently unknown.

SIRT6: The function of SIRT6 is to regulate genomic DNA stability and DNA repair (166). SIRT6 has been demonstrated to deacetylate histones, as well as the DNA repair enzyme, DNA polymerase β *in vitro*. Studies using $SIRT6^{-/-}$ mice die prematurely and suffer from severe glucose homeostasis imbalance (166). These $SIRT6^{-/-}$ mice

experience similar pathologies that are found in elderly humans, implicating that SIRT6 may play a role in maintaining organ integrity as mice age.

SIRT7: The SIRT7 protein deaceytlase is the only sirtuin to be completely localized in the nucleolus (167). SIRT7 is a component of the RNA polymerase I transcription mechanism that interacts with histones and functions to promote the transcription of rDNA during transcriptional elongation (167). Studies that caused SIRT7 deletion prevented cell proliferation and induced cell apoptosis (168). Interestingly, SIRT1 works to oppose SIRT7 by negatively regulating RNA polymerase I via deacetylation of TAF168 (168). Table 4 reviews the expression and role of each mammalian sirtuin homolog in regulating different disease states.

Various inhibitors of the HDACs have been employed to examine their role in cell signalling. These inhibitors are specific to the each class of HDAC and have been used extensively for *in vitro* studies (94;99). Small molecules that inhibit the class I/II HDACs are no able to inhibit sirtuins (class III) and vice versa. Trichostatin A is a potent and reversible inhibitor of class I/II HDACs that does not effect class III of HDACs (169). Sirtuins (class III HDACs) are inhibited by nicotinamide via it's ability to condense with the high-energy enzyme, 2'-O-acetyl-ADP-ribose, to reverse the reaction to reform NAD+ as seen in figure 10 (170). In doing so, nicotinamide operates as a classical non-competitive product inhibitor of the forward deacetylation reaction in sir2 proteins. Conversely, splitomicin is believed to mediate sirtuin inhibition by competing for acetylated substrate binding (170). Finally, sirtinol is a cell-permeable 2-hydroxy-1-napthaldehyde derivative that acts as a direct inhibitor of the sirtuin class of histone deacetylases (169).

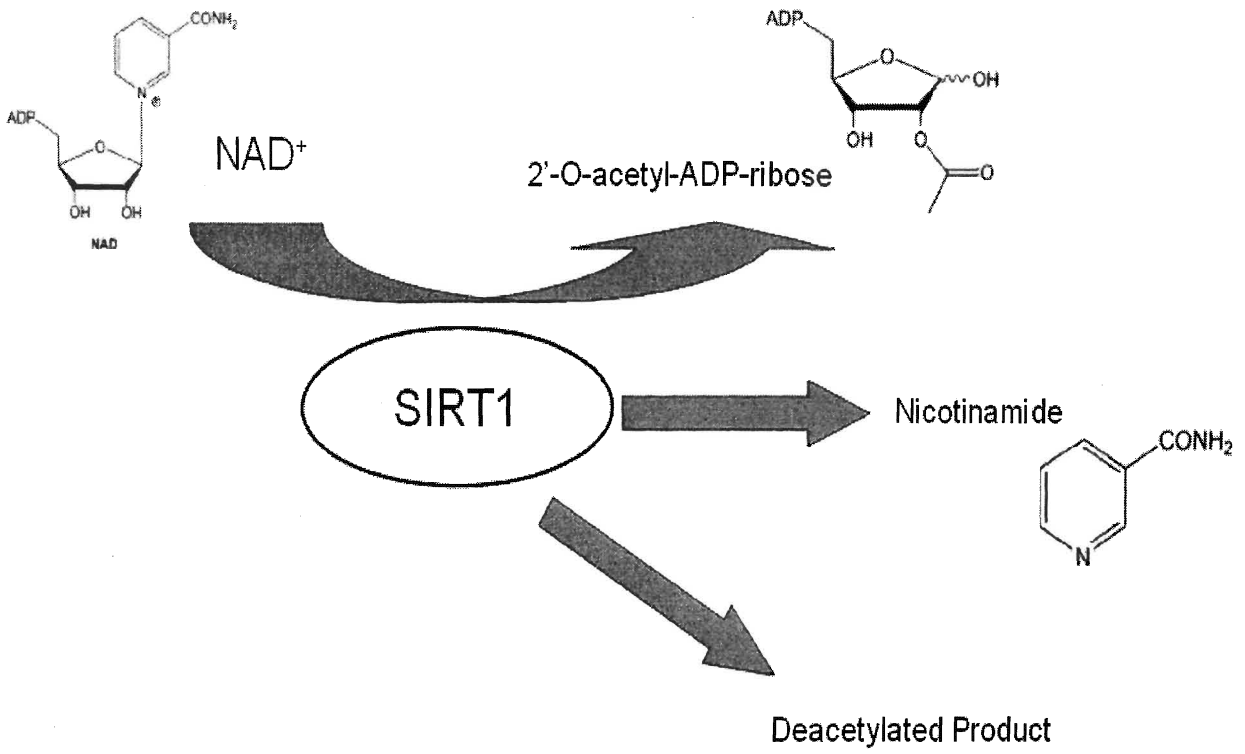


Figure 10: The enzymatic pathway of sirtuin activity (152;171)

1.6l The Antidiabetic Properties of Resveratrol

Resveratrol has been shown to acutely increase glucose uptake in L6 (99) and C2C12 (100) skeletal muscle cell. The proposed mechanism of resveratrol action to increase glucose uptake in skeletal muscle cells is via AMPK activation, which is independent of the insulin signaling cascade (99;100). In addition, in the presence of insulin, cells pretreated with resveratrol were able to improve insulin sensitivity, and promote increased insulin-stimulated activity of PI3-K, Akt , and increased glucose uptake (100).

Table 4: Expression, localization, and function of the mammalian sirtuin deacetylases

SIRT Homolog	Cellular Location	Target Genes	Link with Diseases	Reference
SIRT1	Nuclear/ Cytoplasmic	p53, NFkB, PGC-1α, PPARγ, Foxo	ageing, obesity, insulin resistance, diabetes	Baur et al 2006 (114) Lagouge et al 2006 (119) Ij Youcef et al 2007(145)
SIRT2	Cytoplasmic	α-tubulin	downregulated in human gliomas	Dryden et al 2003 (156)
SIRT3	Mitochondrial	PGC-1α, AceCS2	breast cancer, adaptive thermogenesis	Shi et al 2005 (158)
SIRT4	Mitochondrial	Glutamate dehydrogenase	inhibits amino acid-stimulated insulin secretion	Haigis et al 2006 (159)
SIRT5	Mitochondrial	unknown	unknown	Ij Youcef et al 2007(145)
SIRT6	Nuclear	DNA polβ	age-related diseases	Mostoslavsky et al 2006 (160)
SIRT7	Nuclear	RNA polymerase, Pol1	overexpressed in thyroid cancer and breast cancer	Ford et al 2007 (162) Grummt et al 2003 (161)

It has also been proposed that resveratrol enhances glucose uptake (99) in L6 skeletal muscle cells and insulin sensitivity in C2C12 skeletal muscle cells via a SIRT1-dependent mechanism (172). Studies by Sun et al, 2007 using palmitate induced-insulin resistant C2C12 cells, showed that resveratrol significantly increased insulin-stimulated glucose uptake and improved insulin-stimulated Akt phosphorylation. Resveratrol also enhanced glycogen synthesis in HepG2 cells in a SIRT1-dependent manner, indicating a possible effect of resveratrol to increase insulin action in its target tissues (172).

In addition to *in vitro* studies, resveratrol has been shown to increase insulin sensitivity and survival in mice fed a high-calorie diet *in vivo* (118). Compared to mice fed 60% of calories from fat (HF-diet), both glucose and insulin levels were significantly

diminished in mice that were resveratrol and HF fed. The possible mechanism behind these metabolic effects were believed to be mediated by AMPK activation (118).

Similarly, resveratrol has been shown to possess insulin-like effects in streptozotocin-induced diabetic rats (STZ-DM) (173). Su et al, 2006 observed that resveratrol has hypoglycemic and hypolipidemic effects in STZ-DM rats. The STZ-DM rats that were treated with resveratrol were orally fed resveratrol by gastric intubation at 0.1, 0.25, 0.5, or 0.75 mg/kg body weight, and were sacrificed on day 14. When analyzed, the resveratrol treated STZ-DM rats had their plasma glucose concentration reduced by 25%, and their triglyceride levels reduced by 50% respectively, compared to untreated STZ-DM rats (173). This study also concluded that resveratrol administration ameliorates common diabetic symptoms, such as polyphagia, polydipsia, and weight loss. Furthermore, in STZ-nicotinamide DM rats (a model of moderate insulin-deficient DM), resveratrol treatment significantly reduced insulin secretion and delayed the onset of insulin resistance (173).

In addition, studies by Sun et al, 2007 have shown that resveratrol attenuates high fat diet-induce insulin resistance in skeletal muscle in mice *in vivo*, at a dose of 2.5mg/kg/day, through SIRT1 activation. Mice that were fed a high fat diet exhibited impaired glucose tolerance, but in the presence of resveratrol (2.5mg/kg/day) these mice had glucose disposal curves comparable to control chow-fed mice. In addition, the SIRT1 inhibitor nicotinamide was fed to chow diet mice for 8 weeks. Treatment with nicotinamide significantly increased fat mass, body weight, and fasting blood glucose levels, suggesting that SIRT1 activity may regulate insulin sensitivity *in vivo* (172). Furthermore, one study examined the effects of other SIRT1-activators that are

structurally different from resveratrol. The oral use of the small molecule activators of SIRT1 SRT1460, SRT1720, and SRT2183 in both diet-induced obese mice (DIO) and genetically obese mice (Zucker) demonstrate improved whole-body glucose homeostasis, as well as increased insulin sensitivity compared to their respective control mice (144).

1.7 RATIONAL

Skeletal muscle is accountable for more than 75% of whole body glucose uptake in response to insulin, and thus quantitatively is the most important insulin target tissue (2). Defects in skeletal muscle glucose uptake may lead to decreased insulin sensitivity or insulin resistance, leading to the development of type 2 diabetes (49).

Increased plasma FFAs such as palmitate have been linked to insulin resistance *in vitro* and *in vivo* (34). Studies using L6 and C2C12 skeletal muscle cells have shown that palmitate impairs insulin signalling, increases ceramide production, and induces ser^{307} and $ser^{636/639}$ phosphorylation of IRS-1 (34;35). Several protein kinases, such as GSK3 (40), JNK (41;42), PKC's (66), IKK (34), mTOR (45), and p70 S6K (47) have been implicated in mediating FFA-induced insulin resistance *in vitro* and *in vivo*. These kinases can induce insulin resistance by ser^{307} and $ser^{636/639}$ phosphorylation of the IRS-1 protein, which has been shown to cause impairment in the insulin signalling cascade downstream of the insulin receptor, and lead to a significant reduction in insulin-stimulated glucose uptake (36). Palmitate has not been reported to increase the activity of the protein kinases JNK, mTOR, and p70 S6K in skeletal muscle cells, which will be the focus of our investigation.

Studies *in vivo* have also correlated increased palmitate levels with decreased insulin-stimulated glucose uptake in skeletal muscle and adipose cells (174;175).

Palmitate impairs the ability of GLUT4 to translocate to the plasma membrane and also affects GLUT4 transporter activity, thus preventing glucose entry into insulin sensitive tissues (174). In addition, it has been shown that insulin-induced glucose uptake in skeletal muscle is considerably decreased in obese subjects that have elevated plasma FFA levels (36;176). Thus, investigation of the signalling molecules involved in mediating insulin resistance is very important. The knowledge gained from such studies could be used towards the development of new treatment strategies.

The polyphenol resveratrol has been gaining increased popularity in the scientific community based on its beneficial biological effects (122;158). Recent studies have proposed resveratrol as an antidiabetic agent (99;123). Resveratrol increases glucose uptake in skeletal muscle cells (99) and prevents high fat diet-induced insulin resistance in mice (118). However, the exact mechanism of resveratrol action needs to be elucidated, and therefore further investigation into the antidiabetic effects of resveratrol is required to enhance its potential as a therapeutic treatment for insulin resistance and type 2 diabetes.

1.8 HYPOTHESIS

In this present study it is hypothesized that;

1) Resveratrol ameliorates palmitate-induced skeletal muscle insulin resistance.

2) Resveratrol ameliorates palmitate-induced IRS-1 ser^{307} & $ser^{636/639}$ phosphorylation,

3) Resveratrol ameliorates the palmitate-induced decrease in insulin-stimulated Akt phosphorylation.

4) Resveratrol modulates the expression/activation of the protein kinases JNK, mTOR, and p70 S6K.

5) Sirtuins may be involved in the resveratrol mechanism of action.

6) AMPK may play a role in the resveratrol-mediated effects.

1.9 OBJECTIVES

The primary objectives of this study were;

1) To examine the effects of resveratrol on palmitate-induced insulin resistance in L6 skeletal muscle cells.

2) To examine the effect of resveratrol to modulate the phosphorylation and/or expression of IRS-1.

3) To examine the effect of resveratrol to modulate basal and insulin-induced Akt phosphorylation and/or expression.

4) To investigate the effect of palmitate on the expression and phosphorylation of the protein kinases JNK, mTOR, p70 S6K that have been implicated in mediating insulin resistance.

5) To elucidate the role of sirtuins in mediating resveratrol's effects.

6) To examine possible involvement of AMPK in resveratrol's action.

The information gathered from this study may be used to further the understanding of the mechanisms involved in attenuating skeletal muscle insulin resistance.

CHAPTER 2: METHODOLOGY

2.1 Materials

All tissue culture materials including minimum essential medium (α-MEM), fetal bovine serum (FBS), trypsin and antibiotic were purchased from GIBCO Life Technologies (Burlington, ON). Antibodies against IRS1, ser^{307} phospho-IRS1, ser$^{636/639}$ phospho-IRS1, Akt, phospho-Akt ser^{473}, phospho-Akt thr^{308}, AMPK, phospho-AMPK thr^{172}, JNK, phospho-JNK Thr^{183}/Tyr^{185}, mTOR, phospho-mTOR ser^{2448}, p70-S6K, phospho-p70S6K Thr^{389}, HRP-conjugated anti-rabbit secondary antibody, and ChemiGLOW reagents were purchased from New England Biolabs (Mississauga, ON, Canada). ChemiGLOW reagents was obtained through Thermo-Fischer (Ottawa, ON). Polyvinylidene difluoride (PVDF) membranes, molecular weight protein standards, and electrophoresis reagents were purchased from BioRad (Mississauga, ON). [^{3}H]2-deoxy-D-glucose was purchased from Perkin Elmer (Boston, MA). Chemical inhibitors sirtinol and compound C were purchased from Calbiochem (Gibbstown, NJ). All other chemicals, including cytochalasin B (CB), protein A agarose beads, NaOH, HCl, NaCl, and albumin bovine serum (BSA) were purchased from Sigma (St. Louis, MO). The L6 cells were kind gift of Dr. A. Klip (Hospital for Sick Children, Toronto, ON).

2.2 Buffers and Solutions

A list for the preparation of each buffer and solution used to perform the experiments in the methods & results section will be presented below.

Glucose Uptake Assay:

HBS Washing Buffer: 140mM NaCl, 5mM KCl, 20mM HEPES, 2.5mM $MgS0_4$, 1mM $CaCl_2$, dilute with DD water and fill to 800mL, adjust pH to 7.4 and then fill to 1L.

Total Radioactive Buffer: HBS buffer, dilute 1:1000 hot 2-[^{3}H]deoxy-D-glucose, and 1:1000 cold 2-[^{3}H]deoxy-D-glucose in HBS.

Non-specific radioactive buffer: Use total radioactive buffer, add 1:1000 cytochalasin B.

0.9% NaCl: Add 0.9g NaCl per 100ml DD water.

0.05N NaOH: Add 1ml 5N NaOH to 99ml DD water.

Fatty Acid (FA) Palmitate Solutions:

Palmitic acid (41mg) was dissolved in bubbling 0.1N NaOH (1.6mL) and diluted in prewarmed (45-50°C) 9.7% (W/V) BSA solution (18.4mL).

Cell Lysis:

PBS Washing Buffer: 137mM NaCl, 2.7mM KCl, 1.5mM KH2PO4, 8.1mM Na2HPO4, 0.68mM CaCl2, 0.49mM MgCl2, add water to 1L and adjust pH to 7.4.

SDS Sample Buffer: 62.5mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 0.01% bromophenol blue, add 0.05% β-mercaptoethanol before use.

Cell Lysis Buffer: 20mM Tris (pH 7.5), 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate, 1mM Na3VO4, 1μg/ml leupeptin, add 1mM PMSF before use and chill on ice.

Western Blotting:

1.5M Tris-HCl (pH 8.8): 27.23g Tris base (18.15g/100ml), 80ml deionized water, adjust to pH 8.8 with 6N HCl. Bring to total volume 150ml with deionized water.

0.5M Tris-HCl (pH 6.8): 6g Tris base, 60ml deionized water, adjust to pH 6.8 with 6N HCl and bring total volume to 100ml with deionized water.

Resolving Gel Buffer: 12.3ml DD water, 9.9ml 30% Acrylamide/ Bis solution, 7.5ml 1.5M Tris- Hcl, pH 8.8, 0.3ml 10% w/v SDS. Right before pouring the gel, add 150μl

10% APS (0.01g/ 100µl) made fresh daily and 15µl TEMED and swirl.

Stacking Gel Buffer: 18.3ml DD water, 3.9ml 30% Acrylamide/ Bis solution, 7.5ml 0.5M Tris-HCl, pH 6.8, 0.3ml 10% w/v SDS. Right before pouring the gel, add 150µl 10% APS (0.01g/ 100µl) made fresh daily and 30µl TEMED and swirl.

10x TBS (Tris- buffered saline): 24.2g Tris base, 80g NaCl, adjust pH to 7.6 with HCl. Use at 1x TBS. Dissolve in 1L of water.

Blocking Buffer: 1x TBS, 0.1% tween 20, 5% w/v non-fat dry milk. Dissolved in water.

Primary Antibody Dilution Buffer: 2ml 10x TBS, 18ml water, 1.0g BSA, 20ul Tween- 20.

Wash Buffer TBS/T: 1x TBS and 0.1% Tween- 20.

10x Electrode Running Buffer: 15.15g Tris base, 72g Glycine, 5.0g SDS. Dissolve and bring volume to 500ml with DD water. Do not adjust pH with acid or base. Dilute 50ml of 10x stock with 450ml water before use.

Transfer Buffer: 25mM Tris base, 0.2M glycine, 20% methanol, dissolved in 800mL of water.

2.3 Parental Rat L6 Skeletal Muscle Cells

This line of skeletal muscle cells was derived from 72-hour old rat hindlimb skeletal muscle (177). These cells start of as mononucleated myoblasts, which were cultured in a flask of α-MEM containing 10% FBS. The parental L6 cells then spontaneously differentiate into multinucleated myotubes (in α-MEM containing 2% FBS) over the course of 5-6 days (177). Once fully differentiated, the myotubes express the insulin receptor, insulin receptor substrate-1 (IRS-1), and the insulin-sensitive glucose transport protein GLUT4 (177).

Using the cell culture methodology is beneficial for studies investigating the molecular mechanism of hormones such as insulin, as well as metabolic processes. For example, cells in culture are a homogeneous population of cells with little intracellular space, unlike primary tissue preparation that may contain a large quantity of unrelated supporting tissue. Additionally, the *in vitro* approach is an isolated environment free from external factors that may interfere with cell treatments. Finally, cell culture is efficient when investigating acute and chronic effects of various treatments. They can be passaged multiple times for repetition and validation of different experimental treatments as well.

Specifically, L6 myotubes were selected in this study because palmitate-treated myotubes is an established model of insulin resistance, which we used to test our hypothesis (34;86). Furthermore, skeletal muscle is the predominant target tissue of insulin, and L6 skeletal muscle cells are insulin responsive. L6 myotubes also express the GLUT1 and GLUT4 glucose transport proteins.

2.4 Cell Culture Technique

L6 rat skeletal muscle cells were grown in α-MEM containing 5mM glucose, 10% (v/v) FBS, and 1% (v/v) antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B) until they reached 60-70% confluence. To obtain fully differentiated myotubes, myoblasts were trypsinized and seeded into 6 or 12-well plates in α-MEM medium containing 2% (v/v) FBS with 1% (v/v) antibiotic. This media was replaced every 48h and cells were maintained for 5-6 days until they became fully confluent myotubes. Myotubes were serum-deprived for 16h in all experiments. At the end of treatment, cells were rinsed with HBS followed by a glucose transport assay or cell lysis and western blotting.

2.5 Cell Treatments

Resveratrol was prepared by dissolving the resveratrol powder in 100% absolute ethanol. Stock solutions of insulin were prepared using 2% FBS-containing media. A solution of the inhibitor sirtinol was prepared in sterile DMSO. A vehicle-treated control group was used in parallel with the treatment groups.

Stock palmitate solution was prepared by conjugating palmitate with FA-free BSA (Sigma, St. Louis, MO), as previously reported (34). In brief, sufficient palmitate was dissolved in 0.1N NaOH and diluted in prewarmed (45-50°C) 9.7% (W/V) BSA solution to give a stock concentration of 8mM palmitate solution. The final molar ratio of free palmitate/BSA was 6:1. The final concentration of palmitate is indicated in each figure. A vehicle-treated control group of BSA was used in parallel with each of the treatment groups.

2.6 [3H]2-deoxy-D-glucose Uptake

After cell treatment the myotubes were washed 3 times in HBS at room temperature. Afterwards, cells were incubated with 300μL of total radioactive buffer (10μM) in triplicate, and non-specific radioactive buffer (10μM) for 10min at room temperature. Glucose transport was stopped by washing 3 times with 0.9% NaCl and cell were collected in 1mL of 0.05N NaOH. Cell associated radioactivity was determined by scintillation counting. Protein concentrations were determined using the protein assay protocol from Bio-Rad Laboratories.

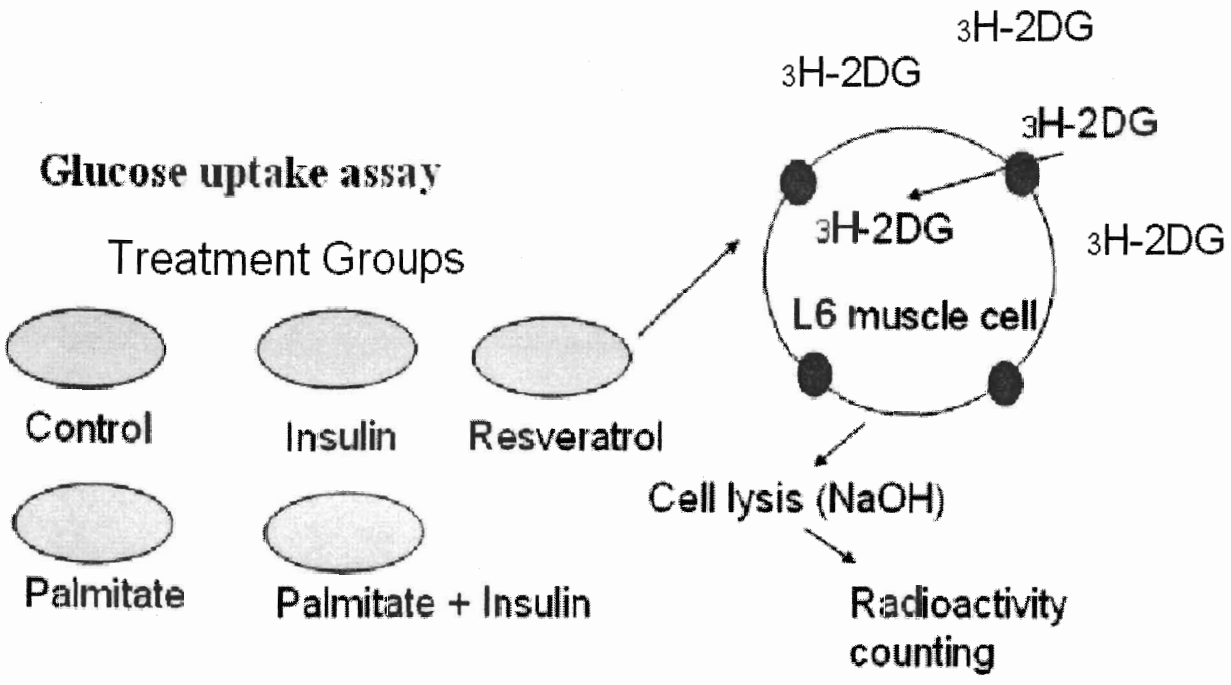


Figure 11: Schematic diagram of the glucose uptake assay protocol

2.7 Cell Lysis

L6 cells were grown in 6-well plates until they were fully confluent, followed by treatments as indicated in the results section. After treatments, cells were washed two times with PBS and placed on ice. PBS was removed and 100μL of lysis buffer was added to each well of the 6-well plates. Cells were lysed and collected into 1.5mL eppendorf tubes, and an equal amount of SDS sample buffer was added to each tube. The cell samples were then boiled for 5min and stored in the freezer at -20°C.

2.8 Protein Assay

Protein assay dye (BioRad) was prepared and filtered for protein concentration determination. BSA protein standards (0, 0.1, 0.2, 0.4, 0.6, 0.8, 0.9, 1.0mg/ml) were used to create a standard protein curve. 10μl of each protein standard and lysed samples were

pipetted into separate wells of a 96-well plate in triplicate. 200μL of protein assay dye was then added into each well. The absorbance was measured using a microplate reader at 595nm, and the final concentration of the protein samples were calculated in Microsoft excel.

2.9 Immunoprecipitation of IRS1

200μg of whole cell lysates were incubated with IRS1 antibody (at a 1:50 volume ratio) overnight a 4°C. This was followed by addition of protein A agarose beads (Sigma, St. Louis, MO), for 1-3 hours at 4°C. The cell lysate was then microcentrifuged, and washed on ice 5 times with cell lysis buffer. The pellet was then resuspended in 20μL of 3x SDS sample buffer and boiled at 100 °C for 5min. 15μL of protein sample were then loaded on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel and analyzed by western blotting.

2.10 Western blotting

At the end of the treatment, the cells were rinsed with PBS, followed by the addition of lysis buffer. The lysate was scraped off and solubilized in 3x SDS sample buffer, followed by separation on a 7-10% SDS-PAGE gel. Samples were then transferred from the gel to a PVDF membrane and blocked for 1h at room temperature with blocking buffer. After blocking, the membrane was incubated with a primary antibody overnight at 4°C. The primary antibody was detected with the appropriate HRP-conjugated secondary antibody, which was exposed to the membrane for 1h in blocking buffer at room temperature. The secondary antibody was visualized by chemiluminescense using ChemiGLOW reagent and FluroChem software (Thermo Fischer).

2.11 Statistical Analysis

Statistical analysis was performed using SPSS v14.0 software. The results are presented as mean ± SEM of at least 3 separate experiments using a student's independent sample T-test or the one-way analysis of variance (ANOVA). Statistical significance was assumed at $P<0.05$.

CHAPER 3: RESULTS

3.1 Resveratrol restores insulin-stimulated glucose uptake in palmitate treated cells.

We examined the effect of the free fatty acid palmitate on insulin stimulated glucose uptake in L6 myotubes. Acute (30min) stimulation of the myotubes with 100nM insulin resulted in a significant increase in 2-[^{3}H]deoxy-D-glucose uptake (175±4.76%, compared to untreated control, $P<0.01$, Fig 12). Incubation with 0.2mM palmitate for 16 hours significantly decreased insulin-stimulated 2-[^{3}H]deoxy-D-glucose uptake (112 ± 9.15% of control, $P<0.01$, Fig 12), indicating insulin resistance. In the presence of resveratrol (25μM), palmitate-treated cells showed significant restoration of insulin-stimulated 2-[^{3}H]deoxy-D-glucose uptake (140 ± 7.14%, $P<0.05$, Fig 12). These data indicate that the negative effect of palmitate on insulin-stimulated glucose uptake is largely ameliorated in the presence of resveratrol. Incubation with palmitate or resveratrol alone did not alter basal glucose uptake compared to control cells (92.5 ± 5.5%, and 92 ± 4.7% of control respectively, Fig 12).

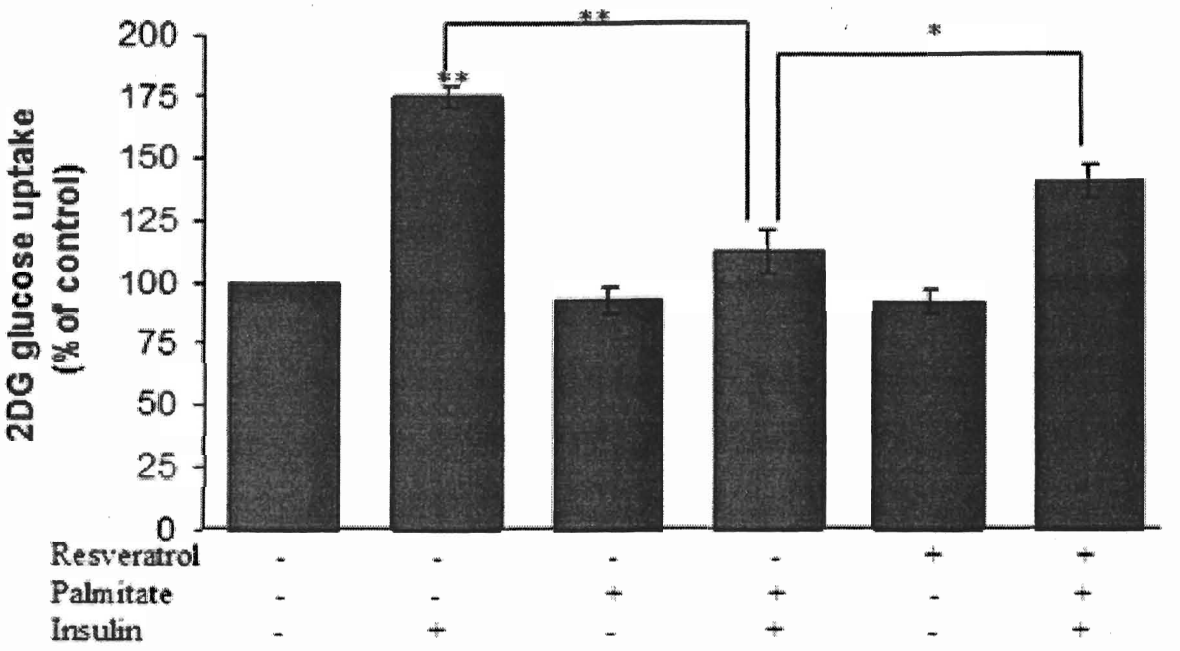


Figure 12: Effects of the fatty acid palmitate and the polyphenol resveratrol on glucose uptake in L6 myotubes. L6 myotubes were incubated with or without 0.2 mM palmitate in the presence or the absence of 25μM resveratrol for 16 h at 37°C, followed by stimulation with or without 100nM insulin for 30 minutes. [^{3}H]2DG uptake was preformed as indicated in the method section. The results are the mean ± SE of 4-8 independent experiments, each performed in triplicate and expressed as a percent of control (*P<0.05, **P<0.01).

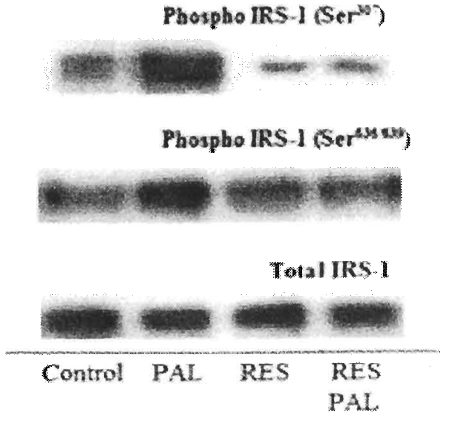
3.2 Resveratrol prevents palmitate-induced ser307 & ser636/639 phosphorylation of IRS1

Once insulin resistance was established at the glucose uptake level, we examined the effects of palmitate and resveratrol downstream of the insulin receptor. We first investigated the effects of palmitate on IRS-1 expression and phosphorylation. Previous studies using L6 skeletal muscle cells *in vitro* and rats muscle *in vivo* have shown that increased ser^{307} & ser$^{636/639}$ phosphorylation of IRS-1 impairs insulin signalling and leads

to insulin resistance (49;178). Using a phospho-specific antibody we examined phosphorylation of the IRS-1 protein on ser^{307} and $ser^{636/639}$ residues.

Myotubes that were treated with palmitate (0.4mM) for 16h demonstrated a significant increase in ser^{307} & $ser^{636/639}$ phosphorylation of IRS-1 (149 ± 18% of control, $P<0.05$, and 152 ± 10.3% of control, $P<0.05$ respectively, Fig 13A, 12B). Treatment of the cells with 25μM resveratrol for 16h had no significant effect on the basal ser^{307} & $ser^{636/639}$ phosphorylation levels of IRS-1 (101 ± 7.3% of control, and 105 ± 9.1% of control, respectively). However, resveratrol completely abolished the palmitate-induced increase in ser^{307} & $ser^{636/639}$ phosphorylation of IRS-1 (114 ± 7.6% of control, $P< 0.05$, and 103 ± 6.3% of control, $P<0.05$ respectively, Fig 13A, 12B). The total levels of IRS-1 did not change significantly with any treatment (PAL: 101 ± 10.5% of control, RES: 99 ± 6.2% of control, RES+PAL: 98 ± 3.6% of control, Fig 12A, 12B). In summary, these results clearly indicate that ser^{307} & $ser^{632/639}$ phosphorylation of IRS-1 is significantly increased by palmitate and resveratrol abolishes this effect.

A)



B)

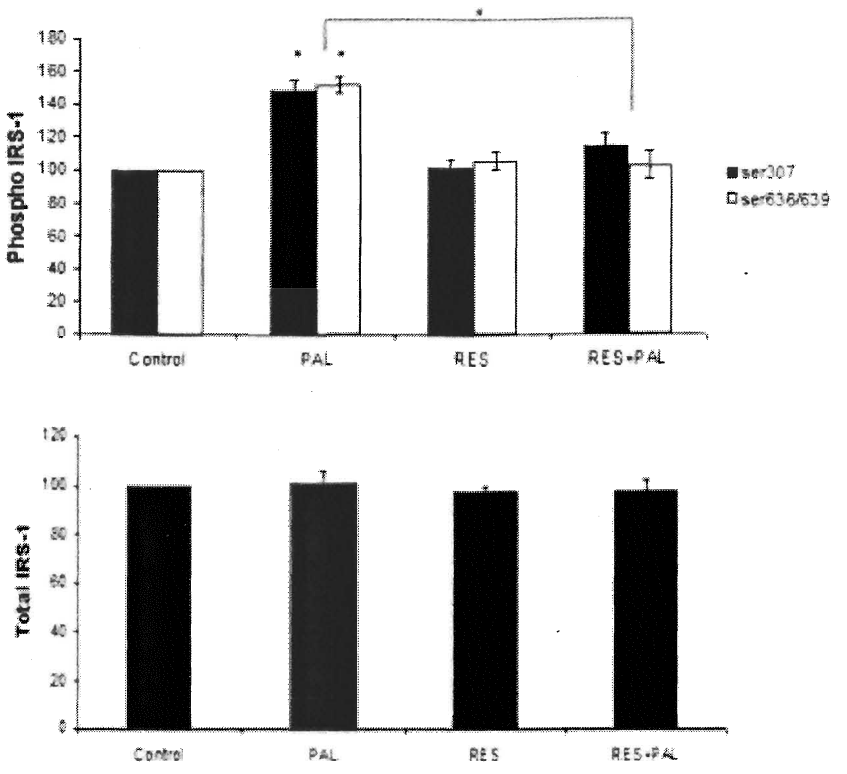


Figure 13: Effects of resveratrol and palmitate on IRS-1 expression and ser^{307} & ser$^{636/639}$ phosphorylation. A) Myotubes were incubated with or without 0.4mM palmitate in the presence or absence of 25μM resveratrol for 16h. After treatment the cells were lysed and IRS-1 was immunoprecipitated as indicated in the methods section. SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognize total or phosphorylated IRS-1 (ser^{307} and ser$^{636/639}$). A representative immunoblot is shown. B) The densitometry of the bands expressed in arbitrary units was calculated for total and phosphorylated (ser^{307} & ser$^{636/639}$) IRS-1 using Alpha innotech software. The values are the mean ± SE of three separate experiments (*P<0.05).

3.3 Resveratrol restores insulin-stimulated Akt phosphorylation in palmitate treated myotubes

Next, we examined the effect of palmitate in the absence or presence of resveratrol on insulin-stimulated Akt phosphorylation and expression. Myotubes that were treated with palmitate for 16h demonstrated a significant decrease in insulin stimulated Akt phosphorylation on both ser^{473} and thr^{308} residues (PAL+INS: 65 ± 8.1%

of INS, $P<0.05$, and 57 ± 7.3% of INS, $P<0.05$, respectively, Fig 14A-B), which is in agreement with other studies (35). However, in the presence of resveratrol, the decline in insulin-stimulated Akt phosphorylation on ser^{473} and thr^{308} seen with palmitate was prevented (RES+PAL+INS: 87 ± 10.5% of INS, $P<0.05$, and 91 ± 9.8% of INS, $P<0.05$, respectively, Fig 14A-B). Resveratrol had no effect on insulin-stimulated Akt phosphorylation (Ser^{473} INS: 214 ± 5.6% of control, and RES+INS: 218 ± 6.2% of control respectively, and on Thr^{308} INS: 210 ± 7.1% of control, and RES+INS: 207 ± 8.0% of control, Fig 14C-D) Treatment of the cells with palmitate or resveratrol alone had no significant effect on the basal phosphorylation (Ser^{473}: 101 ± 10.2% of control, and 98.3 ± 8.4% of control respectively, and on Thr^{308}: 102 ± 4.3% of control, and 99.2 ± 5.1% of control respectively, Fig 14C-D) The total levels of Akt were not significantly changed with any treatment (INS: 104 ± 5.2% of control, PAL: 98 ± 6.3% of control, RES: 97 ± 4.9% of control, RES+INS: 105 ± 7.4% of control, PAL+INS: 89±2.6% of control and RES+PAL+INS: 94±4.5% of control, fig 14).

3.4 Palmitate does not affect JNK expression and phosphorylation

After we established that chronic treatment with palmitate induces ser^{307} & $ser^{636/639}$ phosphorylation of IRS-1, we attempted to determine the signalling molecules that may mediate it. JNK is a serine/threonine kinase that can be activated by many environmental stresses, such as radiation, growth factors, cytokines like tumor necrosis factor alpha (TNFα) or interleukin-1β (IL-1β), and elevated FFA (54). Phosphorylation of JNK at Thr^{183}/Tyr^{185} has been highly correlated with increased JNK activity (179). Activated, JNK has been shown to induce ser^{307} phosphorylation of IRS-1 in liver, muscle, and adipose tissue (62). In addition, phosphorylated JNK (Thr^{183}/Tyr^{185}) was

increased in rats fed a diet high in saturated fat (64). Given that palmitate is a saturated fat, the possibility existed that palmitate increased JNK phosphorylation and/or expression.

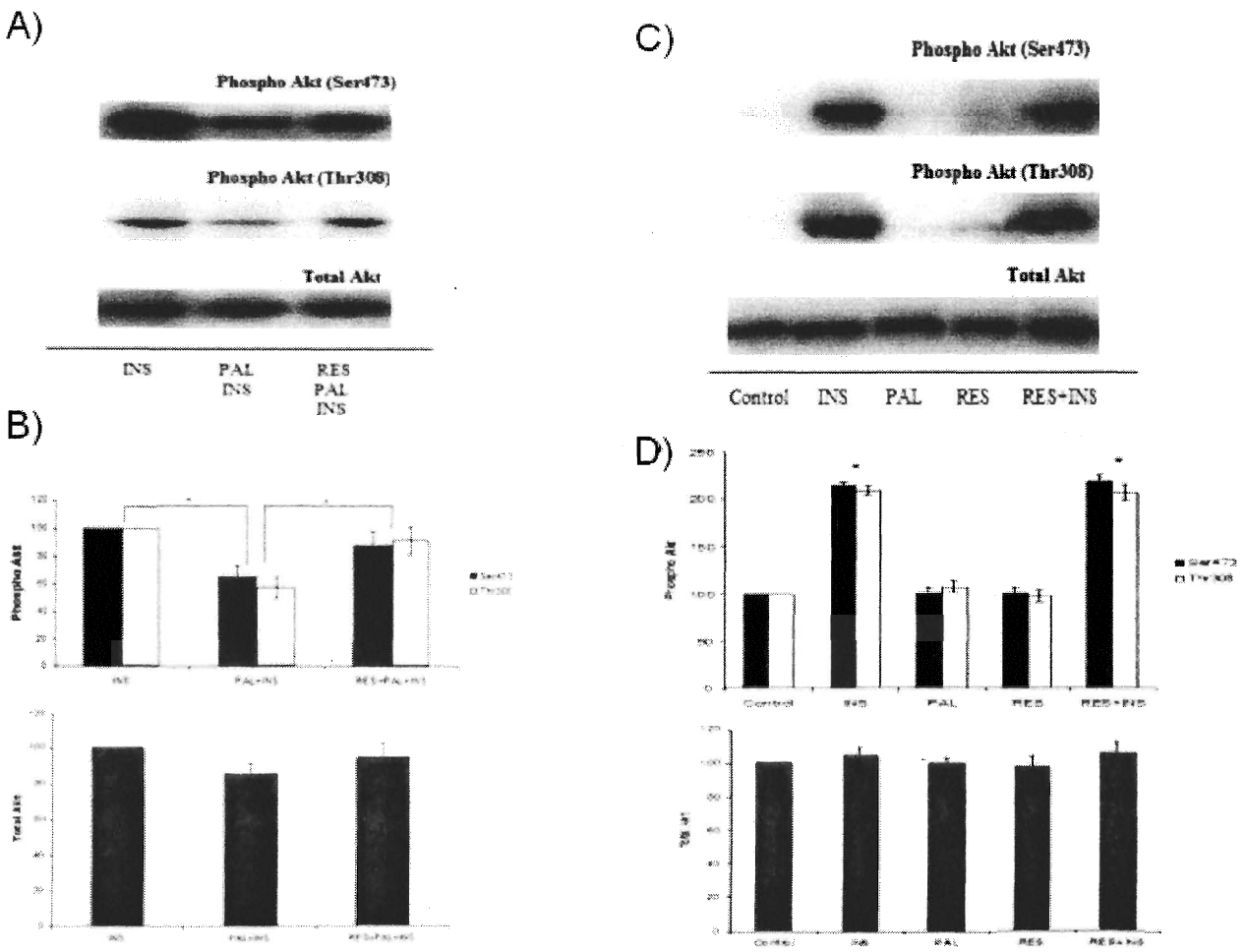


Figure 14: Effects of resveratrol and palmitate on insulin-stimulated Akt phosphorylation and expression. A,C) Myotubes were incubated with or without 0.75 mM palmitate in combination with or without 10 μM resveratrol for 16h. Myotubes were subsequently stimulated with 100 nM of insulin for 10 minutes. The cells were lysed and SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognizes total or phosphorylated Akt (ser^{473} and thr^{308}). A representative immunoblot is shown. B,D) The densitometry of the bands expressed in arbitrary units was calculated for total and phosphorylated Akt (ser^{473} & thr^{308}) using Alpha innotech software. Values are means ± SE of four separate experiments (*$P<0.05$).

Figure 15A-B showed that palmitate for 16h (109.2 ± 5.27% of control) had no effect on JNK phosphorylation and expression. Exposure of the cells to palmitate for 2h, 6h, or 12h had no effect on phospho-JNK as well (Fig 15C). In addition, palmitate had no significant effect on total levels of JNK at 16h (101.1 ± 4.82% of control, Fig 15A-B). Acute insulin stimulation (100nM, for 30min) was used as a positive control (Fig 15A-B), since it is well documented to stimulate JNK phosphorylation. Indeed acute stimulation with insulin resulted in 310 ± 74.5%, $P<0.01$ in JNK phosphorylation compared to control. Acute insulin treatment had no effect on the total levels of JNK (112.7 ± 3.71% of control, Fig 15). 25μM resveratrol for 16h had no effect on the phosphorylation or expression of JNK (96 ± 5.7% of control, and 103 ± 3.9% of control respectively, Fig 15D-E). These findings suggest that JNK is not involved in palmitate-induced insulin resistance in L6 cells.

3.5 Resveratrol prevents palmitate-induced phosphorylation of mTOR in L6 myotubes

Chronic activation of the serine/threonine kinase mTOR has been shown to impair insulin action in L6 myotubes and 3T3-L1 adipocytes (56;180). Furthermore, mTOR is known to become activated by hyperinsulinemia (38), and decrease glucose uptake through ser^{307} phosphorylation of IRS-1 in muscle, liver, and adipose tissue of mice (56). To examine if mTOR involved in palmitate-induced insulin resistance, myotubes were incubated with palmitate in the presence or absence of resveratrol for 16h and total and phosphorylated levels of mTOR were examined.

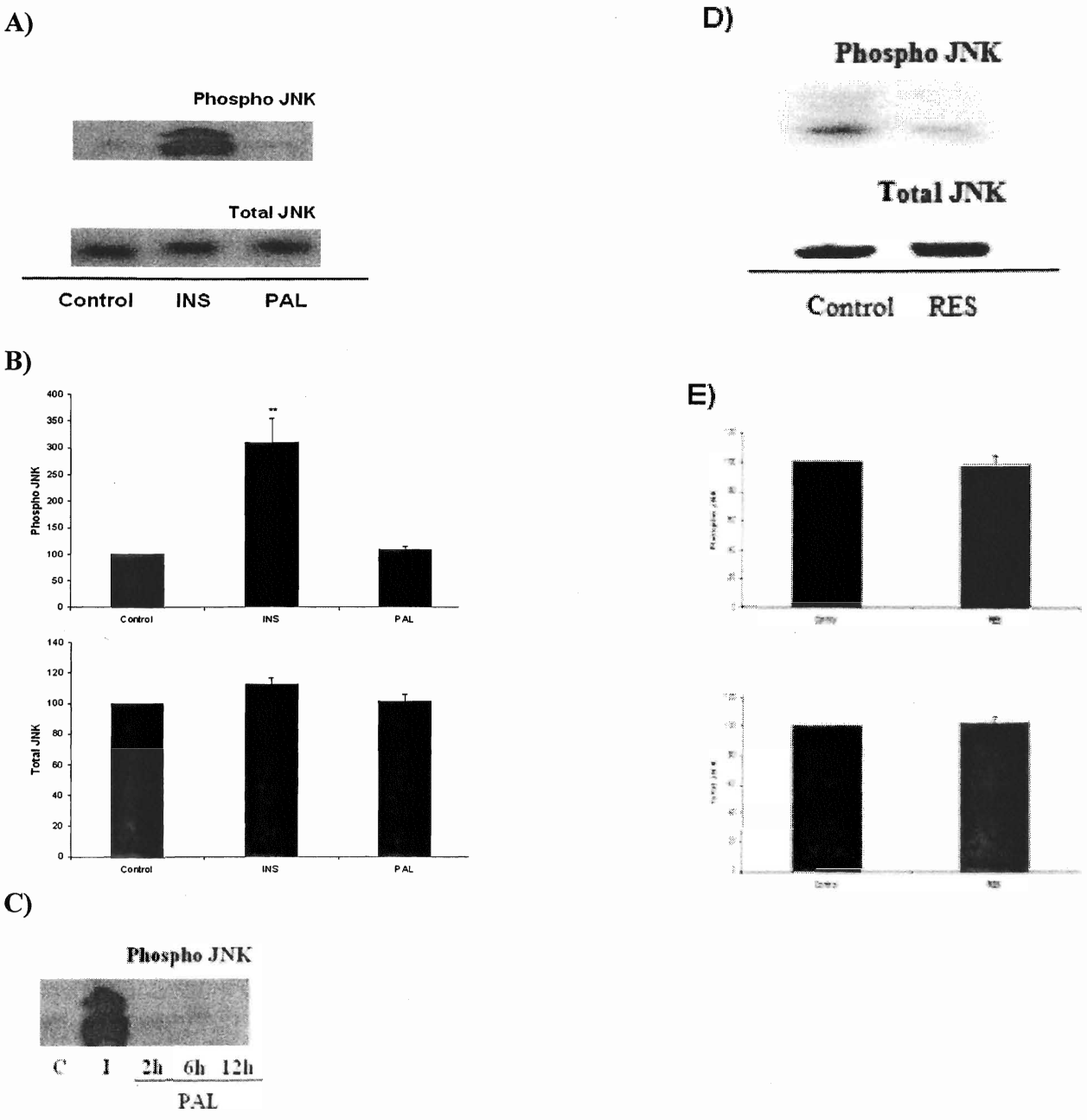


Figure 15: Effect of palmitate on JNK expression and phosphorylation. A,C,D) Myotubes were treated with 0.4 mM palmitate for 2h, 6h, 12h, and 16h. Another group of cell were treated with 25μM resveratrol for 16h or 100 nM of insulin for 10min. The cells were lysed and SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognize total JNK or phosphorylated JNK (Thr^{183}/Tyr^{185}). A representative immunoblot is shown. B,E) The densitometry of the bands expressed as arbitrary units was calculated for total and phosphorylated (Thr^{183}/Tyr^{185}) JNK (fig 14b) using Alpha innotech software. Values are means ± SE of four separate experiments (**P<0.01).

Palmitate treated cells (0.4mM) for 16h showed a significant increase in mTOR phosphorylation (149 ± 2.51% of control, $P<0.05$, Fig 16A-B). However, myotubes that were pretreated with 25μM resveratrol showed complete abolishment in mTOR phosphorylation (83.3 ± 7.07% of control, $P<0.05$, Fig 16A-B). Resveratrol alone had no effect on the phosphorylation of mTOR (96 ± 4.8% of control, Fig 16C-D) The total levels of mTOR were not significantly changed with any treatment: palmitate 104 ± 7.13% of control, resveratrol 103 ± 4.3% of control, and palmitate in the presence of resveratrol 16h 97.1 ± 6.07% of control (Fig 16).

3.6 Resveratrol prevents palmitate-induced phosphorylation of p70 S6K in L6 myotubes

To examine weather p70 S6K plays a role in palmitate-induced insulin resistance we measured its expression and phosphorylation. Myotubes treated with 0.4mM palmitate for 16h showed a significant increase in p70 S6K phosphorylation (155 ± 10.5% of control, Fig 17A-B). Cells treated with palmitate in the presence of 25μM resveratrol for 16h demonstrated complete abolishment in p70 S6K phosphorylation (92 ± 9.0% of control, Fig 17A-B). Resveratrol alone had no effect on the phosphorylation of p70 S6K (98 ± 4.5% of control, Fig 17C-D). There were no significant changes in the total levels of p70 S6K with any treatments: palmitate 16h 95.9 ± 5.9% of control, resveratrol 102 ± 6.3% of control or palmitate in the presence of resveratrol 16h 95.4 ± 13.4% of control (Fig 17).

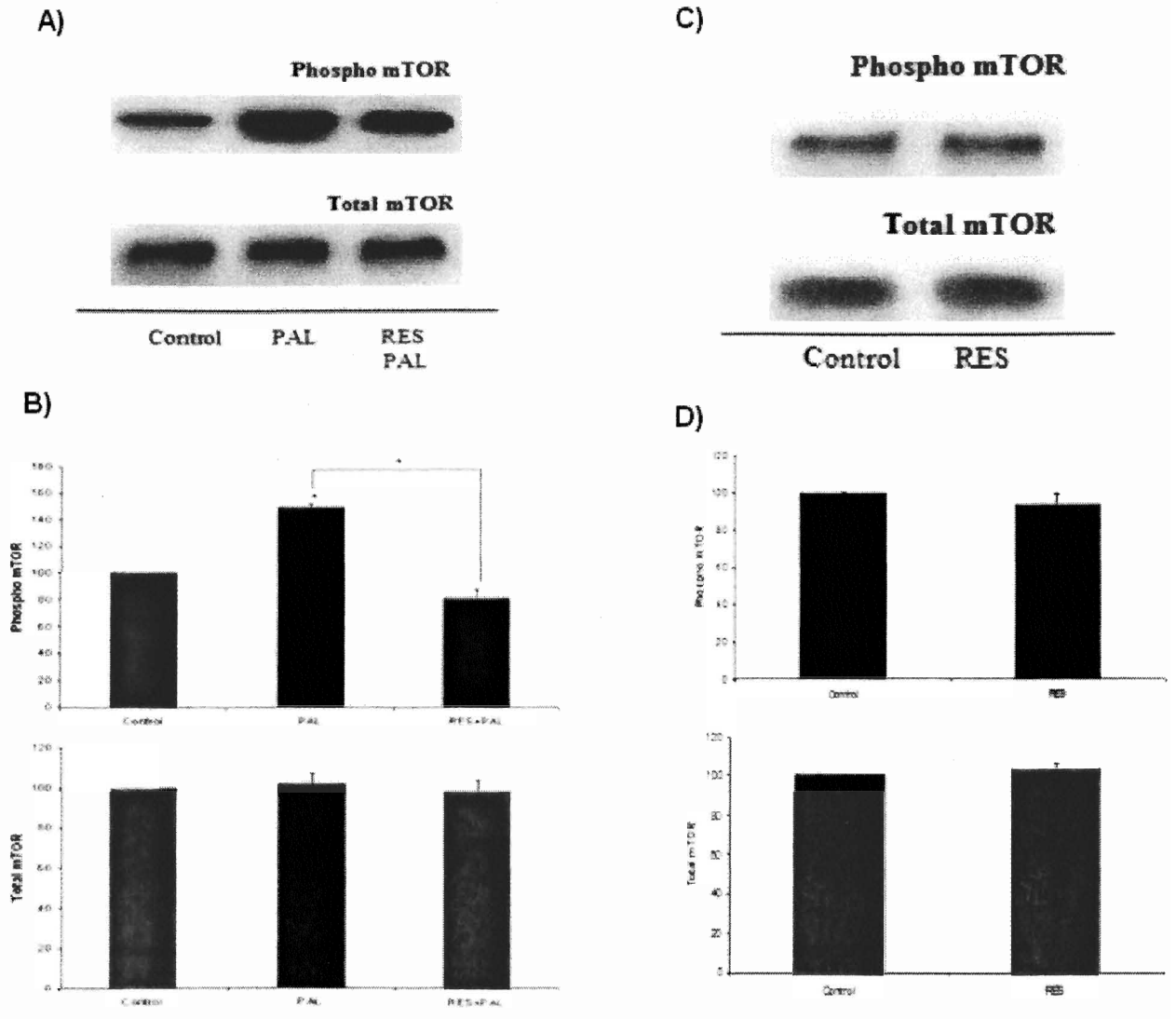


Figure 16: Effects of resveratrol and palmitate on mTOR phosphorylation and expression. A,C) Myotubes were incubated with 0.4 mM palmitate in the presence or absence of 25μM resveratrol for 16 h. The cells were lysed and SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognizes total or phosphorylated mTOR (ser^{2448}). A representative immunoblot is shown. B,D) The densitometry of the bands expressed in arbitrary units was calculated for total and phosphorylated (ser^{2448}) mTOR using Alpha innotech software. Values are means ± SE of three separate experiments (*P<0.05).

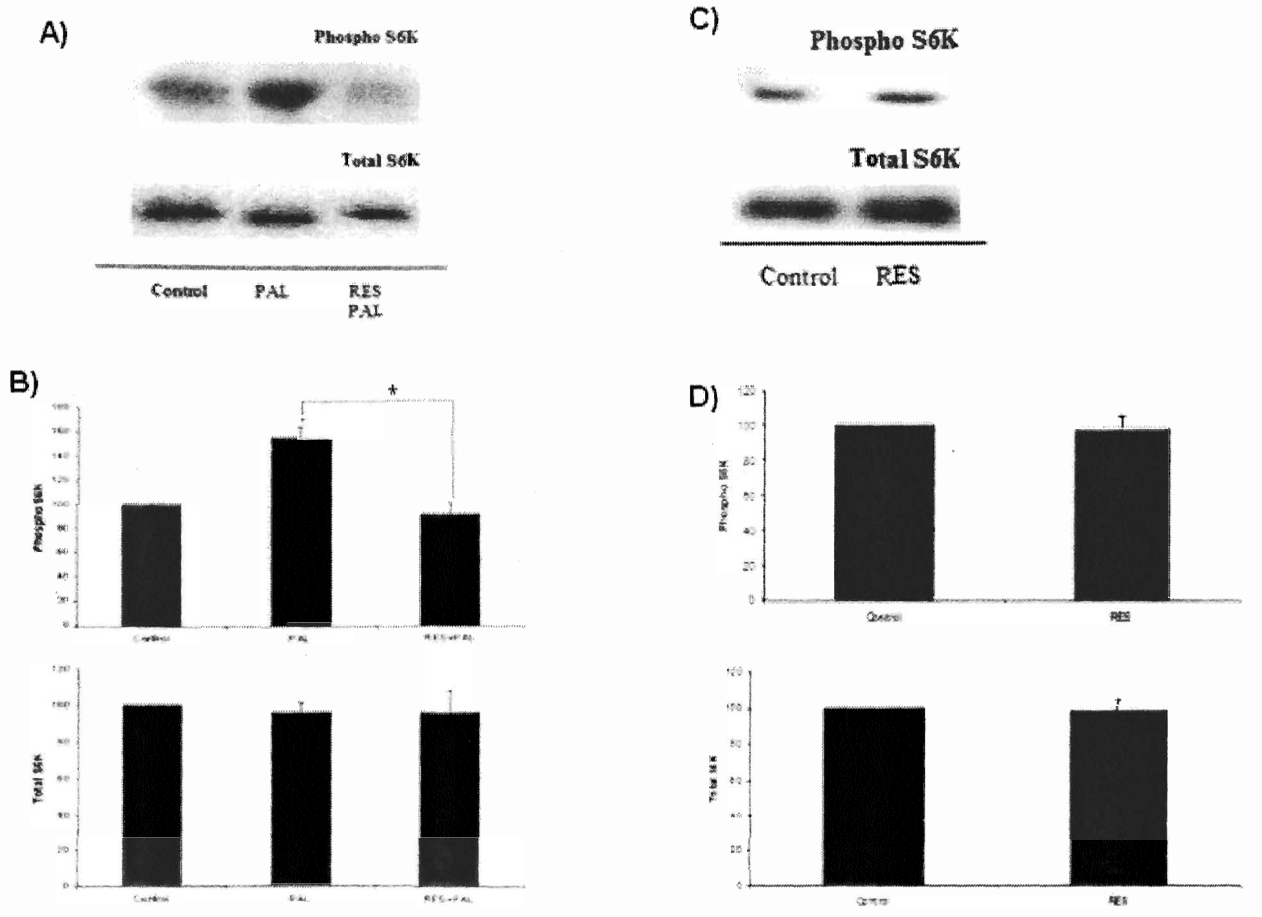


Figure 17: Effects of resveratrol and palmitate on p70 S6K phosphorylation and expression. A,C) Myotubes were incubated with 0.4 mM palmitate in the presence or absence of 25μM resveratrol for 16 h. The cells were lysed and SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognizes total p70-S6K and phosphorylated (Thr^{389}) p70 S6K. A representative immunoblot is shown. B,D) The densitometry of the bands expressed in arbitrary units was calculated for total and phosphorylated (Thr^{389}) p70 S6K using Alpha innotech software. Values are means ± SE of three separate experiments ($*P<0.05$).

3.7 The effect of resveratrol to prevent phosphorylation of IRS-1, mTOR, and S6K is sirtuin-dependent

Resveratrol has been established to activate sirtuins, the NAD^+ dependent deacetylases. Recent studies have suggested SIRT1 to play a role in insulin sensitivity,

energy metabolism, and glucose homeostasis (144). To investigate whether resveratrol mediates its effects through sirtuins we used sirtinol, a potent inhibitor of sirtuins.

Myotubes were treated as before, but in the presence of 50μM sirtinol, the ability of resveratrol to prevent palmitate-induced phosphorylation of IRS-1 (ser^{307} and $ser^{636/639}$) and phosphorylation/activation of mTOR, and p70 S6K was blocked (131 ± 5.2% of control $P<0.05$, 167 ± 9.2% of control $P<0.01$, and 160 ± 5.4% of control $P<0.05$, respectively, Fig 18). Sirtinol alone had no effect on total or phosphorylated levels of IRS-1, mTOR, and p70-S6K (*Total*: 103 ± 2.5% of control, 102 ± 1.3% of control, and 103 ± 2.3% of control, *phospho*: 108 ± 23% of control, 90 ± 9.7% of control, and 52 ± 4.6% of control, respectively, Fig 18).

3.8 The effect of resveratrol to restore insulin-stimulated Akt phosphorylation in palmitate treated myotubes is sirtuin-dependent

In addition, we examined the effect of the sirtuin inhibitor on the expression and phosphorylation of Akt on both ser^{473} and thr^{308} residues. Sirtinol alone (50μM, 16h) had no effect on Akt phosphorylation (108 ± 11.9% of control, Fig 19C-D) or expression (107 ± 8.1% of control, Fig 19C-D). However, in the presence of sirtinol the ability of resveratrol to prevent the palmitate-induced decrease in insulin-stimulated Akt phosphorylation was abolished (67 ± 10.5% of insulin alone, $P<0.05$, and 61 ± 7.7% of INS, $P<0.05$, respectively, Fig 19A-B). These data indicated that sirtuins may mediate resveratrol's effect to restore insulin-stimulated Akt phosphorylation.

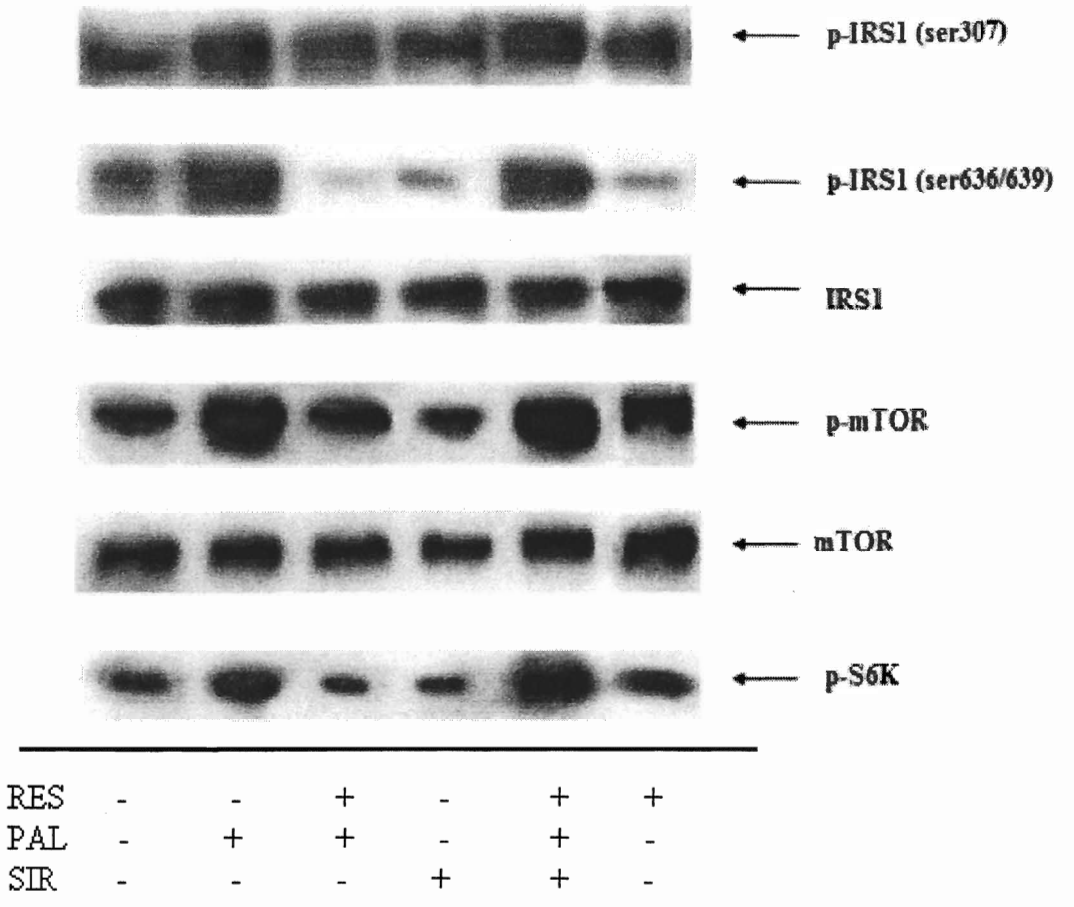


Figure 18: Effects of sirtinol, resveratrol, and palmitate on the phosphorylation and expression of IRS-1, mTOR, and p70 S6K. Myotubes were treated with or without 50μM sirtinol in the presence or absence of 25μM resveratrol and/or 0.4mM palmitate for 16h. The cells were lysed and SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognizes total IRS-1, mTOR, p70-S6K, and phosphorylated IRS-1 (ser^{307} and $ser^{636/639}$), mTOR (ser^{2248}), p70-S6K (Thr^{389}). A representative immunoblot of these separate experiments is shown.

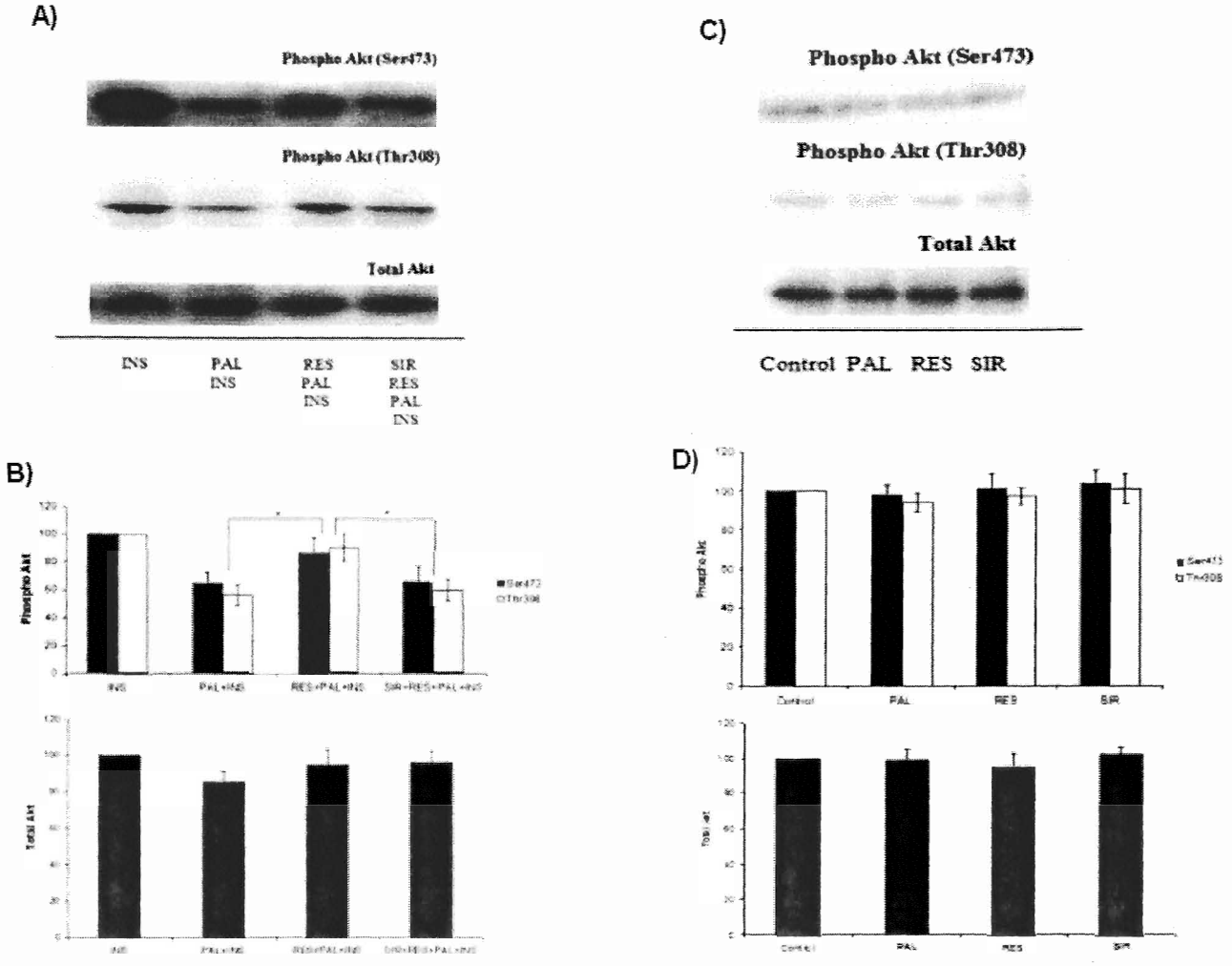


Figure 19: Effects of sirtinol, resveratrol, and palmitate on Akt phosphorylation and expression. A,C) Myotubes were treated with or without 50μM sirtinol for 16h, in the presence or absence of 10μM resveratrol and/or 0.4mM palmitate for 16h. The cells were then stimulated with 100nM insulin for 10min, followed by cell lysis. SDS-PAGE was performed, proceeded by immunobloting with specific antibodies that recognize total or phosphorylated (ser^{473} and thr^{308}) Akt. A representative immunoblot is shown. B,D) The densitometry of the bands expressed in arbitrary units was calculated for total and phosphorylated (ser^{473} & thr^{308}) Akt using Alpha innotech software. Values are means ± SE of four separate experiments (*$P<0.05$).

3.9 The effect of resveratrol to stimulate AMPK phosphorylation is sirtuin-dependent

Previous studies by our group indicate that resveratrol acutely increases glucose uptake through increased AMPK phosphorylation that may be mediated by sirtuins (99). In the present study, we investigated the chronic (16h) effect of resveratrol on the phosphorylation of AMPK, and we examined whether sirtuins are upstream of AMPK using nicotinamide a well established inhibitor of sirtuins.

Chronic resveratrol treatment (25μM, 16h) significantly increased the phosphorylation of AMPK (RES: 173 ± 14.2% of control, $P<0.05$, Fig 20A-B), even in the presence of 0.4mM palmitate (RES+PAL: 169 ± 4.8% of control, $P<0.05$, Fig 20A-B). However, in the presence of the sirtuin inhibitor, nicotinamide (6mM, 16h), resveratrol-stimulated AMPK-phosphorylation was completely abolished (NIC+RES: 106 ± 7.0% of control, and NIC+RES+PAL: 110 ± 9.2% of control, Fig 20A-B) indicating that sirtuins may be upstream of AMPK. Palmitate alone had no effect on the phosphorylation of AMPK (104 ± 6.4% of control, Fig 20C-D). The expression levels of AMPK did not change with any treatment (RES: 107 ± 6.7% of control, NIC: 101 ± 9.2% of control, PAL: 98 ± 4.7% of control, NIC+RES: 104 ± 5.6% of control, RES+PAL: 98 ± 5.9% of control, and NIC+RES+PAL: 99 ± 8.1% of control, Fig 20).

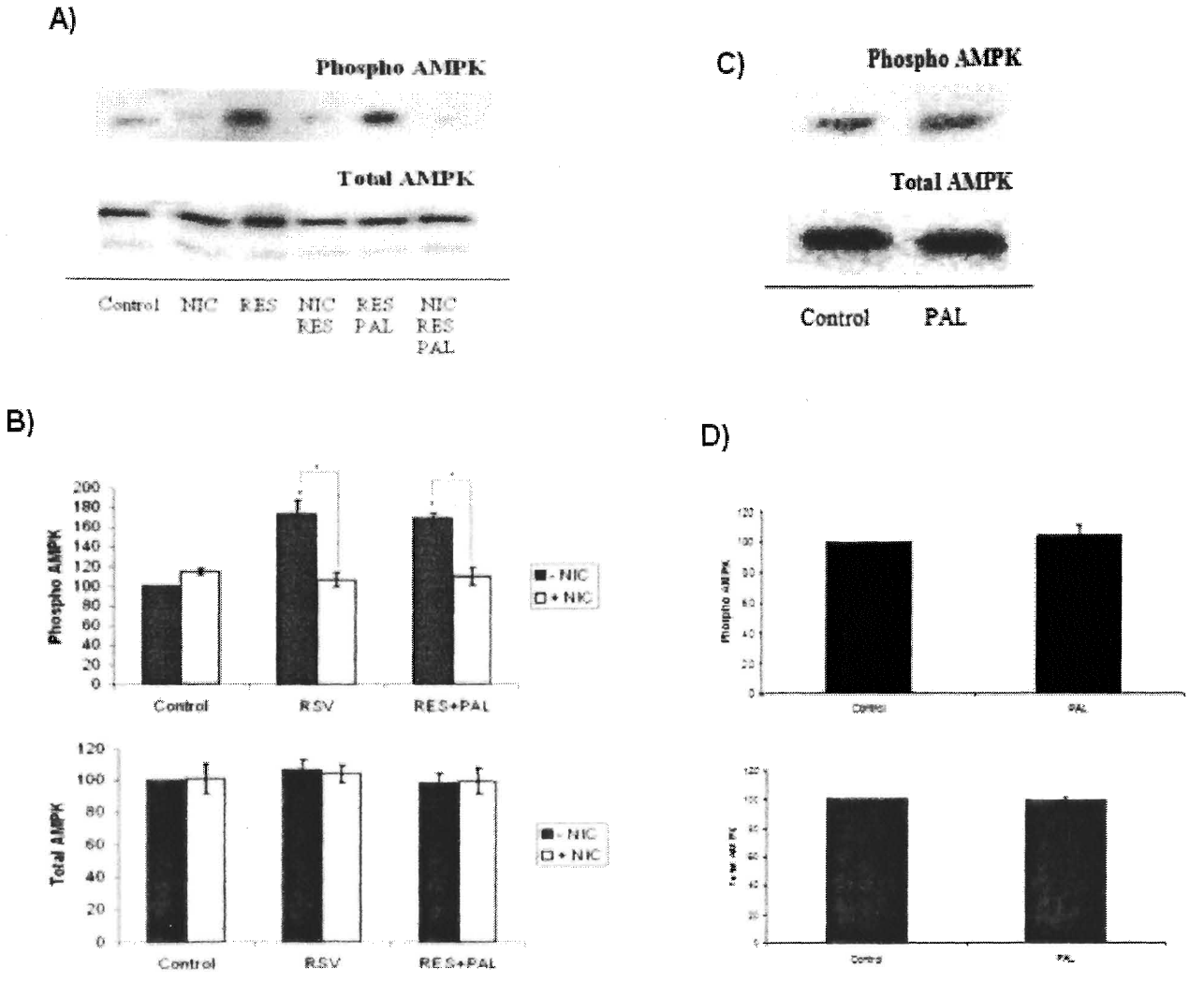


Figure 20: Effects of nicotinamide, resveratrol, and palmitate on the phosphorylation and expression of AMPK. A,C) Myotubes were incubated with or without 6mM nicotinamide for 16h in the presence or absence of 25μM resveratrol or 0.4mM palmitate for 16 h. The cells were lysed and SDS-PAGE was performed, followed by immunobloting with specific antibodies that recognizes total and phosphorylated (Thr^{172}) AMPK. A representative immunoblot is shown. B,D) The densitometry of the bands expressed in arbitrary units was calculated for total and phosphorylated (Thr^{172}) AMPK using Alpha innotech software. Values are means ± SE of three separate experiments (*$P<0.05$).

CHAPTER 4: DISCUSSION

4.1 Resveratrol Ameliorates Palmitate-Induced Insulin Resistance

Insulin resistance is highly correlated with obesity and elevated FFAs, which are risk factors for the development of type 2 diabetes (36). Thus, therapeutic approaches to prevent FFA-induced insulin resistance would be beneficial. Previously, the FFA palmitate has been shown to induce insulin resistance in skeletal muscle cells (86;87). Indeed, our findings demonstrated that there was a significant reduction in insulin-stimulated glucose uptake in myotubes that were chronically treated for 16h with 0.2mM palmitate (fig 12). However, palmitate had no effect on basal glucose uptake.

Given that skeletal muscle is the main target for glucose disposal in the post-prandial state, and is the major target of insulin action, it is expected that new interventions improving glucose transport in this tissue would be desirable. Recently, it was demonstrated that resveratrol attenuates high-fat diet-induced insulin resistance in skeletal muscle tissue of mice *in vivo* (172). Additionally, studies in C2C12 skeletal muscle cells have shown that treatment with resveratrol have significantly increased insulin-stimulated glucose uptake and improved insulin signalling by modulating molecules downstream of the insulin receptor (100). These studies are in agreement with our findings, in that resveratrol attenuates palmitate-induced insulin resistance, through the modulation of key signalling molecules in L6 skeletal muscle cells.

We have shown that chronic 16h treatment with 25μM resveratrol did not alter the basal levels of glucose uptake, although it has been established that acute treatment with resveratrol increases glucose uptake *in vitro* (99;100). In the presence of 25μM resveratrol, palmitate-treated cells showed significant restoration of insulin-stimulated

glucose uptake (fig 12), indicating that resveratrol prevents palmitate-induced insulin resistance.

4.2 Elucidating the Mechanism of Action of Resveratrol in Insulin-Resistant Myotubes

Palmitate-induced phosphorylation of IRS-1 on its ser^{307} & $ser^{636/639}$ residues is known to mediate insulin resistance in skeletal muscle (49;53). Serine phosphorylated IRS-1 makes a poor substrate for the insulin receptor, thereby inhibiting its downstream effects (37). Our results show that myotubes chronically treated for 16h with 0.4mM palmitate cause a significant increase in IRS-1 ser^{307} and $ser^{636/639}$ phosphorylation (fig 13). However, in the presence of 25μM resveratrol palmitate-induced ser^{307} & $ser^{636/639}$ phosphorylation of IRS-1 was completely abolished.

Downstream of IRS-1 in the insulin signalling cascade is the serine/threonine kinase Akt. This protein is required for insulin-stimulated GLUT4 translocation and facilitated entry of glucose into skeletal muscle (1;29). Studies using small interfering RNA (siRNA) to deplete Akt in 3T3-L1 cultured adipocytes have demonstrated that insulin-stimulated glucose uptake is completely abolished, indicating that it is a key player in insulin signalling (33). We examined the phosphorylation of Akt at ser^{473} and thr^{308}, which are required for Akt activation (181). Inhibition of insulin-stimulated phosphorylation of Akt was observed in L6 myotubes that were chronically incubated with 0.75mM palmitate for 16h, (fig 14), as seen by other reports (35). However, Akt phosphorylation was restored in myotubes that were treated with palmitate in the presence of 10μM resveratrol for 16h (fig 14). The concentration of palmitate and resveratrol used in this experiment are in close proximity to other studies that

demonstrated improved glucose uptake and phosphorylation of Akt in insulin resistant C2C12 skeletal muscle cells (172). However, these concentrations of resveratrol are far greater (10-1000 times) that the maximal plasma concentrations found in human subjects following oral administration of resveratrol, due to its rapid metabolism (182).

JNK is an insulin-responsive serine/threonine kinase that has been implicated in mediating insulin resistance via ser^{307} phosphorylation of IRS-1 (62). Sustained activation of JNK was seen in primary mouse hepatocytes and pancreatic β-cells of high-fat diet-induced insulin resistant mice and streptozotocin (STZ)-induced diabetic mice. In contrast, mice with a targeted mutation at the JNK locus that abolished JNK expression, proved to be resistant to high fat-induced insulin resistance (62). We examined the effect of 0.4mM palmitate on phosphorylation of JNK and found that it had no effect (fig 15), suggesting that FFA-induced phosphorylation of JNK may be tissue specific.

The mTOR and p70 S6K pathways have also been suggested to regulate FFA-induced insulin resistance (79). The activity of mTOR and p70 S6K are increased in the liver and skeletal muscle of rats that are fed high-fat diet (47;183), indicating that this signalling pathway may play a role in inducing insulin resistance *in vivo* (79). *In vitro* the effect of mTOR-induced insulin resistance is largely reversed using the mTOR inhibitor rapamycin (56). Similarly, knockout mice that have a S6K1 deletion exhibit decreased IRS-1 phosphorylation on ser^{307} and $ser^{636/639}$ subjected to a high-fat diet when compared to their wild-type counterparts (56).

The phosphorylation of mTOR and p70 S6K was significantly increased when myotubes were chronically treated for 16h with 0.4mM palmitate (fig 16, 17). Similar results were observed in obese rats fed a high-fat diet, which lead to increased mTOR

activity (180). This observation provides a possible mechanism of action for palmitate to induce skeletal muscle insulin resistance, via activation of this pathway. However, myotubes chronically treated with palmitate in the presence of 25μM resveratrol demonstrated complete inhibition of palmitate-induced mTOR and p70 S6K phosphorylation. This suggests that resveratrol may ameliorate palmitate-induced insulin resistance through inhibition of the mTOR and p70 S6K pathway, since both protein kinases are known to serine phosphorylate IRS-1. (fig 16, 17).

4.3 Resveratrol Ameliorates Palmitate-Induced Insulin Resistance through Sirtuins

Our results demonstrated that the effect of resveratrol to prevent palmitate-induced ser^{307} and $ser^{636/639}$ phosphorylation of IRS-1 was inhibited in the presence of 50μM sirtinol a sirtuin inhibitor, which suggests that sirtuins may play a role in resveratrol's mechanism of action (fig 18). In a recent study by Zhang et al, 2007 it was shown in rat hepatocytes that SIRT1 is associated with the IRS proteins, and regulates insulin-stimulated tyrosine phosphorylation of IRS-1 via deacetylation (154). It is possible that the observed resveratrol effects in our study are mediated by a similar activation of sirtuins which is associated with IRS-1 to prevent its ser^{307} and $ser^{636/639}$ phosphorylation. However, the effects of resveratrol to inhibit mTOR and p70 S6K was also abolished by sirtinol (fig 18). Thus, the possibility exists that resveratrol goes through sirtuins to inhibit mTOR and p70 S6K phosphorylation, thereby ameliorating ser^{307} and $ser^{636/639}$ phosphorylation of IRS-1.

Previously, it has been reported that resveratrol mediates its antidiabetic properties, in part through activation of SIRT1 (123;144) and AMPK (100;118). Recently, it has been shown that AMPK is upstream of SIRT1 in skeletal myoblasts that underwent

glucose restriction (184). In contrast, SIRT1 was recently shown to be an upstream regulator of AMPK to control hepatocyte lipid metabolism (94). It is therefore not clear whether sirtuins are upstream or downstream of AMPK. In agreement with previous studies by our group and other (94), we observed that the resveratrol-stimulated phosphorylation of AMPK was completely abolished in the presence of the sirtuin inhibitor, nicotinamide (fig 20). This suggests in our cells that sirtuins are upstream of AMPK, as has been suggested in hepatocytes (94).

In addition, we observed that the ability of resveratrol to restore insulin-stimulated Akt phosphorylation in the presence of palmitate was attenuated by sirtinol (fig 19). This suggests that the effect of resveratrol to improve Akt phosphorylation may also be dependent on sirtuins.

Taken together, we hypothesized that resveratrol exerts its effect through the AMPK and sirtuins to attenuate palmitate-induced phosphorylation of mTOR and p70 S6K (as shown in figure 21). In addition, sirtuin inhibition through the use of sirtinol prevents the ability of resveratrol to block palmitate-induced mTOR and p70 S6K activation, thereby allowing subsequent serine phosphorylation of IRS-1.

In conclusion, our results indicate that palmitate induces skeletal muscle insulin resistance that was correlated with increased phosphorylation of mTOR and p70 S6K and increased ser^{307} and $ser^{636/639}$ phosphorylation of IRS-1. We observed that resveratrol inhibits palmitate-induced insulin resistance in L6 myotubes. In addition, resveratrol significantly decreased ser^{307} and $ser^{636/639}$ phosphorylation of IRS-1 and restored the insulin-stimulated phosphorylation of Akt.

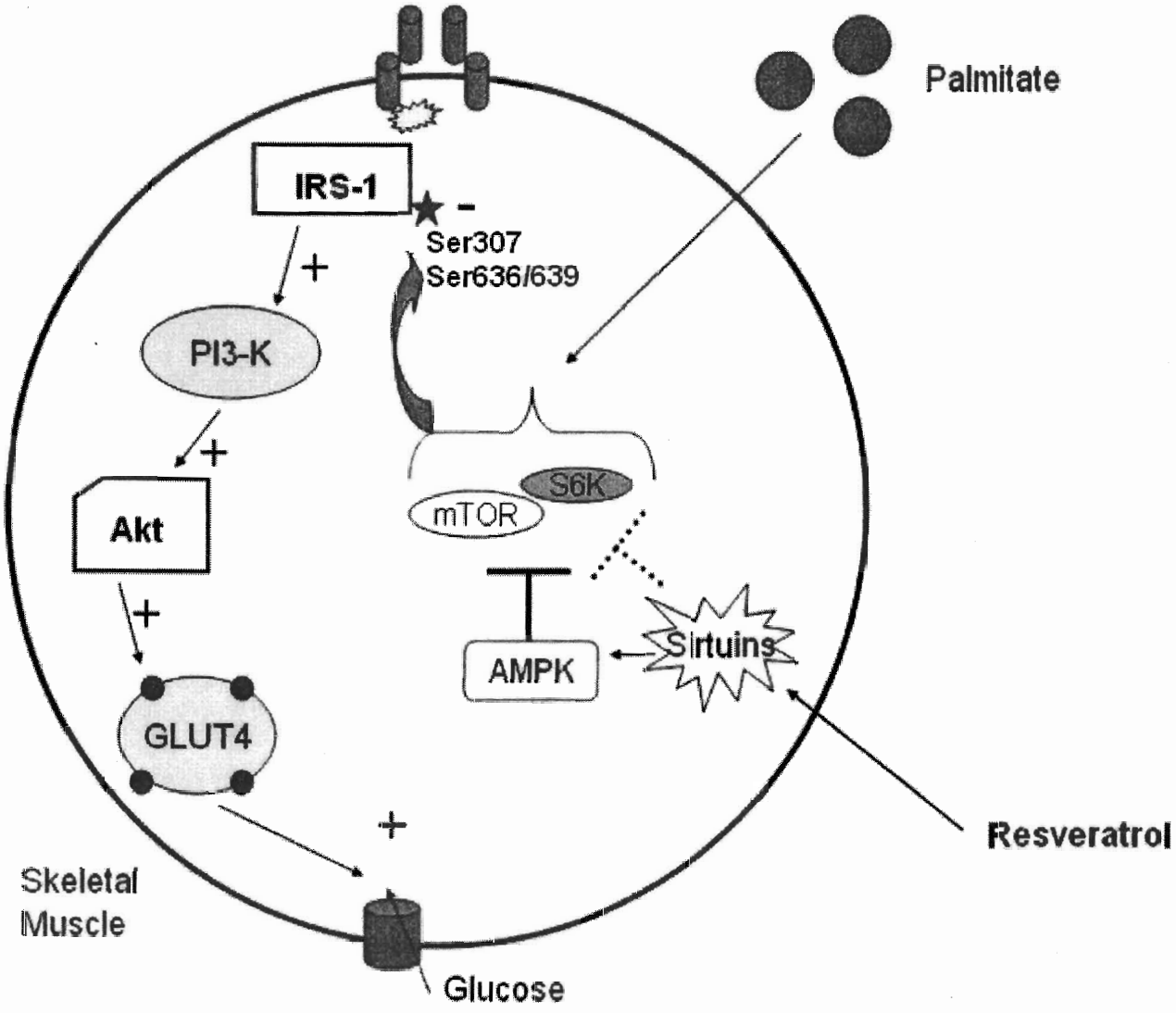


Figure 21: Proposed mechanism of resveratrol action to prevent palmitate-induced insulin resistance in L6 skeletal muscle cells

On the other hand, the beneficial effect of resveratrol was attenuated in the presence of the sirtuin inhibitors sirtinol and nicotinamide. Therefore, a possible mechanism of action of resveratrol may involve sirtuins and AMPK. These findings implicate resveratrol as a promising therapeutic agent in the attenuation of insulin resistance and type 2 diabetes.

4.4 Significance of the Present Study

The prevalence of T2DM is increasing and is expected to affect 380 million people globally by the year 2025 (Canadian Diabetes Association). In addition, insulin resistance and T2DM is associated with the development of other disease pathologies such as cardiovascular disease and cancer (185). Thus new therapeutic approaches to help prevent and manage insulin resistance could be beneficial to society. As mentioned previously, obesity and the presence of elevated levels of the FFA palmitate, have been reported to mediate insulin resistance in skeletal muscle and liver cells (45;118). Recently, resveratrol was demonstrated to have antidiabetic properties (144) and to have the ability to ameliorate FFA-induced insulin resistance in mice (118).

This study has shown that palmitate can induce skeletal muscle insulin resistance in rat L6 skeletal muscle cells. Palmitate significantly inhibited insulin-stimulated glucose uptake and increased the phosphorylation of IRS-1 on numerous serine residues (ser^{307} and $ser^{636/639}$), thereby preventing downstream insulin signalling. In addition, palmitate significantly increased the phosphorylation of mTOR and p70-S6K. Importantly, resveratrol prevents the palmitate-induced increase in phosphorylation of IRS-1, mTOR, p70-S6K, and restored insulin-stimulated glucose uptake in L6 myotubes. These findings are novel and provide compelling evidence for the use of resveratrol in the possible treatment of insulin resistance and T2DM.

4.5 Future Directions

The role of the AMPK pathway in mediating resveratrol's effects could be examined more extensively by using the AMPK inhibitor compound C or siRNA approach. If AMPK is indeed playing a significant role in the mechanism of action of

resveratrol we would expect that inhibition of AMPK would counteract the positive effects of resveratrol on palmitate-induced insulin resistant in L6 myotubes. Similarly, future studies could utilize specific sirtuin inhibitors or siRNA approach to investigate the role of sirtuins. Based on recent reports (94;154;172) that indicate SIRT1 plays a role in insulin signalling and insulin sensitivity, it is valid to examine the role of the SIRT1 sirtuin homolog on resveratrol-mediated attenuation of palmitate-induced insulin resistance in L6 myotubes. If SIRT1 is inhibited we would expect that the ability of resveratrol to ameliorate palmitate-induced insulin resistance would be abolished.

To provide stronger evidence for the involvement of mTOR and p70-S6K as mediators of palmitate-induced insulin resistance in our cells, the mTOR inhibitor rapamycin could be utilized. It is expected that rapamycin would have the same effect as resveratrol to ameliorate palmitate-induced insulin resistance in L6 myotubes. A similar approach would be to use siRNA against mTOR and/or p70 S6K.

This study used an established model of palmitate-induced skeletal muscle insulin resistance *in vitro* (34;35). The methodology of this study was based on examining the phosphorylation and expression of proteins in the insulin signalling cascade (IRS-1, Akt, JNK, mTOR, p70 S6K) and AMPK using immuno-blotting. Our study only examined the ser^{307} and $ser^{636/639}$ phosphorylation sites of IRS-1. Future research could study the effect of skeletal muscle insulin resistance on the ser^{612} and ser^{1101} phosphorylation sites of IRS-1, as well as tyrosine phosphorylation levels of IRS-1. We would predict that tyrosine phosphorylation of IRS-1 is decreased in the presence of palmitate and that resveratrol can attenuate this effect. Furthermore, the results from immuno-blotting measure the phosphorylation levels of proteins on specific residues that are correlated with protein

activity. To provide stronger evidence of protein activation, *in vitro* activity assays could be incorporated into future studies.

GSK3 and PKC have been implicated in the modulation of skeletal muscle insulin resistance and could be examined in the future. In addition, the IKK/NFκβ and immune response pathway has also been highly correlated to FFA-induced insulin resistance (186). Toll-like receptor 4 (TLR4) are a family of receptors that play a role in the innate immune system by activating pro-inflammatory signalling pathways, such as IKK and NFκβ in response to microbial pathogens (187). Recent studies have shown that the activity of TLR4 is increased in lipid-infused mice and obese individuals (187). It has also been reported that resveratrol can directly inhibit NFκβ activity (160). Thus, future studies could examine the effect palmitate on the immune response pathway in L6 myotubes. We would expect that resveratrol could attenuate insulin resistance mediated through the TLR4 and IKK/NFκβ pathway.

Future research should also focus on *in vivo* studies. A rat/mouse model of FFA-induced insulin resistance could be used to examine the physiological effects of resveratrol and its mechanism of action. Additionally, primary tissue cultures of adipose, liver, and skeletal muscle tissue could be obtained from these *in vivo* models to assess the effects of resveratrol and FFA in each insulin target tissue.

Reference List

1. Ver MR,CH&QJ: Insulin Signalling Pathways Regulating Translocation of GLUT4. *Current Medical Chemistry* 5:159-165, 2007

2. Taniguchi,CM, Emanuelli,B, Kahn,CR: Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol* 7:85-96, 2006

3. Gillespi K. Type 1 Diabetes: Pathogenesis and Prevention. Canadian Medical Association (175), 165-170. 2007.
Ref Type: Magazine Article

4. Plum,L, Belgardt,BF, Bruning,JC: Central insulin action in energy and glucose homeostasis. *J Clin Invest* 116:1761-1766, 2006

5. Herman,MA, Kahn,BB: Glucose transport and sensing in the maintenance of glucose homeostasis and metabolic harmony. *J Clin Invest* 116:1767-1775, 2006

6. Inzucchi,SE: Oral antihyperglycemic therapy for type 2 diabetes: scientific review. *JAMA* 287:360-372, 2002

7. Zhou,G, Myers,R, Li,Y, Chen,Y, Shen,X, Fenyk-Melody,J, Wu,M, Ventre,J, Doebber,T, Fujii,N, Musi,N, Hirshman,MF, Goodyear,LJ, Moller,DE: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108:1167-1174, 2001

8. Hallsten,K, Virtanen,KA, Lonnqvist,F, Sipila,H, Oksanen,A, Viljanen,T, Ronnemaa,T, Viikari,J, Knuuti,J, Nuutila,P: Rosiglitazone but not metformin enhances insulin- and exercise-stimulated skeletal muscle glucose uptake in patients with newly diagnosed type 2 diabetes. *Diabetes* 51:3479-3485, 2002

9. Medina,RA, Owen,GI: Glucose transporters: expression, regulation and cancer. *Biol Res* 35:9-26, 2002

10. Ishiki,M, Klip,A: Minireview: recent developments in the regulation of glucose transporter-4 traffic: new signals, locations, and partners. *Endocrinology* 146:5071-5078, 2005

11. Ganguly,A, Devaskar,SU: Glucose transporter isoform-3-null heterozygous mutation causes sexually dimorphic adiposity with insulin resistance. *Am J Physiol Endocrinol Metab* 294:E1144-E1151, 2008

12. Leturque,A, Brot-Laroche,E, Le,GM, Stolarczyk,E, Tobin,V: The role of GLUT2 in dietary sugar handling. *J Physiol Biochem* 61:529-537, 2005

13. Ploug,T, Ralston,E: Exploring the whereabouts of GLUT4 in skeletal muscle (review). *Mol Membr Biol* 19:39-49, 2002

14. Zisman,A, Peroni,OD, Abel,ED, Michael,MD, Mauvais-Jarvis,F, Lowell,BB, Wojtaszewski,JF, Hirshman,MF, Virkamaki,A, Goodyear,LJ, Kahn,CR, Kahn,BB: Targeted disruption of the glucose transporter 4 selectively in muscle causes insulin resistance and glucose intolerance. *Nat Med* 6:924-928, 2000

15. Walberg-Henriksson A,&ZR: GLUT4: a key player regulating glucose homeostasis? Insights from transgenic and knockout mice. *Molecular Membrane Biology*205-211, 2007

16. Van,OE, Baron,V, Delahaye,L, Emanuelli,B, Filippa,N, Giorgetti-Peraldi,S, Lebrun,P, Mothe-Satney,I, Peraldi,P, Rocchi,S, Sawka-Verhelle,D, Tartare-Deckert,S, Giudicelli,J: Surfing the insulin signaling web. *Eur J Clin Invest* 31:966-977, 2001

17. Lee,J, Pilch,PF: The insulin receptor: structure, function, and signaling. *Am J Physiol* 266:C319-C334, 1994

18. Kwon,M, Ling,Y, Maile,LA, Badley-Clark,J, Clemmons,DR: Recruitment of the tyrosine phosphatase Src homology 2 domain tyrosine phosphatase-2 to the p85 subunit of phosphatidylinositol-3 (PI-3) kinase is required for insulin-like growth factor-I-dependent PI-3 kinase activation in smooth muscle cells. *Endocrinology* 147:1458-1465, 2006

19. Shepherd,PR, Nave,BT, O'Rahilly,S: The role of phosphoinositide 3-kinase in insulin signalling. *J Mol Endocrinol* 17:175-184, 1996

20. Kahn,CR: Knockout mice challenge our concepts of glucose homeostasis and the pathogenesis of diabetes. *Exp Diabesity Res* 4:169-182, 2003

21. Araujo,EP, De Souza,CT, Gasparetti,AL, Ueno,M, Boschero,AC, Saad,MJ, Velloso,LA: Short-term in vivo inhibition of insulin receptor substrate-1 expression leads to insulin resistance, hyperinsulinemia, and increased adiposity. *Endocrinology* 146:1428-1437, 2005

22. Gharbi,SI, Zvelebil,MJ, Shuttleworth,SJ, Hancox,T, Saghir,N, Timms,JF, Waterfield,MD: Exploring the specificity of the PI3K family inhibitor LY294002. *Biochem J* 404:15-21, 2007

23. Sasaki,T, Suzuki,A, Sasaki,J, Penninger,JM: Phosphoinositide 3-kinases in immunity: lessons from knockout mice. *J Biochem (Tokyo)* 131:495-501, 2002

24. Searl,TJ, Silinsky,EM: LY 294002 inhibits adenosine receptor activation by a mechanism independent of effects on PI-3 kinase or casein kinase II. *Purinergic Signal* 1:389-394, 2005

25. Sugatani,T, Hruska,KA: Akt1/Akt2 and mammalian target of rapamycin/Bim play critical roles in osteoclast differentiation and survival, respectively, whereas Akt is dispensable for cell survival in isolated osteoclast precursors. *J Biol Chem* 280:3583-3589, 2005

26. Shiojima,I, Walsh,K: Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ Res* 90:1243-1250, 2002

27. Vanhaesebroeck,B, Alessi,DR: The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J* 346 Pt 3:561-576, 2000

28. Cong,LN, Chen,H, Li,Y, Zhou,L, McGibbon,MA, Taylor,SI, Quon,MJ: Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endocrinol* 11:1881-1890, 1997

29. Carvalho,E, Rondinone,C, Smith,U: Insulin resistance in fat cells from obese Zucker rats--evidence for an impaired activation and translocation of protein kinase B and glucose transporter 4. *Mol Cell Biochem* 206:7-16, 2000

30. Wang,Q, Somwar,R, Bilan,PJ, Liu,Z, Jin,J, Woodgett,JR, Klip,A: Protein kinase B/Akt participates in GLUT4 translocation by insulin in L6 myoblasts. *Mol Cell Biol* 19:4008-4018, 1999

31. Cho,H, Thorvaldsen,JL, Chu,Q, Feng,F, Birnbaum,MJ: Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 276:38349-38352, 2001

32. Cho,H, Mu,J, Kim,JK, Thorvaldsen,JL, Chu,Q, Crenshaw,EB, III, Kaestner,KH, Bartolomei,MS, Shulman,GI, Birnbaum,MJ: Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). *Science* 292:1728-1731, 2001

33. Jiang,ZY, Zhou,QL, Coleman,KA, Chouinard,M, Boese,Q, Czech,MP: Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. *Proc Natl Acad Sci U S A* 100:7569-7574, 2003

34. Sinha,S, Perdomo,G, Brown,NF, O'Doherty,RM: Fatty acid-induced insulin resistance in L6 myotubes is prevented by inhibition of activation and nuclear localization of nuclear factor kappa B. *J Biol Chem* 279:41294-41301, 2004

35. Powell,DJ, Turban,S, Gray,A, Hajduch,E, Hundal,HS: Intracellular ceramide synthesis and protein kinase Czeta activation play an essential role in palmitate-induced insulin resistance in rat L6 skeletal muscle cells. *Biochem J* 382:619-629, 2004

36. Hulver,MW, Dohm,GL: The molecular mechanism linking muscle fat accumulation to insulin resistance. *Proc Nutr Soc* 63:375-380, 2004

37. Kanety,H, Feinstein,R, Papa,MZ, Hemi,R, Karasik,A: Tumor necrosis factor alpha-induced phosphorylation of insulin receptor substrate-1 (IRS-1). Possible mechanism for suppression of insulin-stimulated tyrosine phosphorylation of IRS-1. *J Biol Chem* 270:23780-23784, 1995

38. Ueno,M, Carvalheira,JB, Tambascia,RC, Bezerra,RM, Amaral,ME, Carneiro,EM, Folli,F, Franchini,KG, Saad,MJ: Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. *Diabetologia* 48:506-518, 2005

39. Gual,P, Le Marchand-Brustel,Y, Tanti,JF: Positive and negative regulation of insulin signaling through IRS-1 phosphorylation. *Biochimie* 87:99-109, 2005

40. Lee,J, Kim,MS: The role of GSK3 in glucose homeostasis and the development of insulin resistance. *Diabetes Res Clin Pract* 77 Suppl 1:S49-S57, 2007

41. Hirosumi,J, Tuncman,G, Chang,L, Gorgun,CZ, Uysal,KT, Maeda,K, Karin,M, Hotamisligil,GS: A central role for JNK in obesity and insulin resistance. *Nature* 420:333-336, 2002

42. Tuncman,G, Hirosumi,J, Solinas,G, Chang,L, Karin,M, Hotamisligil,GS: Functional in vivo interactions between JNK1 and JNK2 isoforms in obesity and insulin resistance. *Proc Natl Acad Sci U S A* 103:10741-10746, 2006

43. Li,Y, Soos,TJ, Li,X, Wu,J, Degennaro,M, Sun,X, Littman,DR, Birnbaum,MJ, Polakiewicz,RD: Protein kinase C Theta inhibits insulin signaling by phosphorylating IRS1 at Ser(1101). *J Biol Chem* 279:45304-45307, 2004

44. Sriwijitkamol,A, Christ-Roberts,C, Berria,R, Eagan,P, Pratipanawatr,T, Defronzo,RA, Mandarino,LJ, Musi,N: Reduced skeletal muscle inhibitor of kappaB beta content is associated with insulin resistance in subjects with type 2 diabetes: reversal by exercise training. *Diabetes* 55:760-767, 2006

45. Mordier,S, Iynedjian,PB: Activation of mammalian target of rapamycin complex 1 and insulin resistance induced by palmitate in hepatocytes. *Biochem Biophys Res Commun* 362:206-211, 2007

46. Carlson,CJ, White,MF, Rondinone,CM: Mammalian target of rapamycin regulates IRS-1 serine 307 phosphorylation. *Biochem Biophys Res Commun* 316:533-539, 2004

47. Um,SH, Frigerio,F, Watanabe,M, Picard,F, Joaquin,M, Sticker,M, Fumagalli,S, Allegrini,PR, Kozma,SC, Auwerx,J, Thomas,G: Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature* 431:200-205, 2004

48. Manning,BD: Balancing Akt with S6K: implications for both metabolic diseases and tumorigenesis. *J Cell Biol* 167:399-403, 2004

49. Le Marchand-Brustel,Y, Gual,P, Gremeaux,T, Gonzalez,T, Barres,R, Tanti,JF: Fatty acid-induced insulin resistance: role of insulin receptor substrate 1 serine phosphorylation in the retroregulation of insulin signalling. *Biochem Soc Trans* 31:1152-1156, 2003

50. Draznin,B: Molecular mechanisms of insulin resistance: serine phosphorylation of insulin receptor substrate-1 and increased expression of p85alpha: the two sides of a coin. *Diabetes* 55:2392-2397, 2006

51. Tanti,JF, Gremeaux,T, Van,OE, Le Marchand-Brustel,Y: Serine/threonine phosphorylation of insulin receptor substrate 1 modulates insulin receptor signaling. *J Biol Chem* 269:6051-6057, 1994

52. Ruiz-Alcaraz,AJ, Liu,HK, Cuthbertson,DJ, McManus,EJ, Akhtar,S, Lipina,C, Morris,AD, Petrie,JR, Hundal,HS, Sutherland,C: A novel regulation of IRS1 (insulin receptor substrate-1) expression following short term insulin administration. *Biochem J* 392:345-352, 2005

53. Reynoso,R, Salgado,LM, Calderon,V: High levels of palmitic acid lead to insulin resistance due to changes in the level of phosphorylation of the insulin receptor and insulin receptor substrate-1. *Mol Cell Biochem* 246:155-162, 2003

54. Prada,PO, Zecchin,HG, Gasparetti,AL, Torsoni,MA, Ueno,M, Hirata,AE, Corezola do Amaral,ME, Hoer,NF, Boschero,AC, Saad,MJ: Western diet modulates insulin signaling, c-Jun N-terminal kinase activity, and insulin receptor substrate-1ser307 phosphorylation in a tissue-specific fashion. *Endocrinology* 146:1576-1587, 2005

55. Aguirre,V, Uchida,T, Yenush,L, Davis,R, White,MF: The c-Jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser(307). *J Biol Chem* 275:9047-9054, 2000

56. Le,BO, Petroulakis,E, Paglialunga,S, Poulin,F, Richard,D, Cianflone,K, Sonenberg,N: Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2. *J Clin Invest* 117:387-396, 2007

57. Tremblay,F, Brule,S, Hee,US, Li,Y, Masuda,K, Roden,M, Sun,XJ, Krebs,M, Polakiewicz,RD, Thomas,G, Marette,A: Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. *Proc Natl Acad Sci U S A* 104:14056-14061, 2007

58. Ciaraldi,TP, Nikoulina,SE, Bandukwala,RA, Carter,L, Henry,RR: Role of glycogen synthase kinase-3 alpha in insulin action in cultured human skeletal muscle cells. *Endocrinology* 148:4393-4399, 2007

59. Woodgett,JR, Cohen,P: Multisite phosphorylation of glycogen synthase. Molecular basis for the substrate specificity of glycogen synthase kinase-3 and casein kinase-II (glycogen synthase kinase-5). *Biochim Biophys Acta* 788:339-347, 1984

60. Liberman,Z, Eldar-Finkelman,H: Serine 332 phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 attenuates insulin signaling. *J Biol Chem* 280:4422-4428, 2005

61. Summers,SA, Kao,AW, Kohn,AD, Backus,GS, Roth,RA, Pessin,JE, Birnbaum,MJ: The role of glycogen synthase kinase 3beta in insulin-stimulated glucose metabolism. *J Biol Chem* 274:17934-17940, 1999

62. Solinas,G, Naugler,W, Galimi,F, Lee,MS, Karin,M: Saturated fatty acids inhibit induction of insulin gene transcription by JNK-mediated phosphorylation of insulin-receptor substrates. *Proc Natl Acad Sci U S A* 103:16454-16459, 2006

63. az-Delfin,J, Morales,M, Caelles,C: Hypoglycemic action of thiazolidinediones/peroxisome proliferator-activated receptor gamma by inhibition of the c-Jun NH2-terminal kinase pathway. *Diabetes* 56:1865-1871, 2007

64. Zhang,Z, Li,X, Lv,W, Yang,Y, Gao,H, Yang,J, Shen,Y, Ning,G: Ginsenoside Re Reduces Insulin Resistance through Inhibition of JNK and NF-{kappa}B. *Mol Endocrinol* 2007

65. Serra,C, Federici,M, Buongiorno,A, Senni,MI, Morelli,S, Segratella,E, Pascuccio,M, Tiveron,C, Mattei,E, Tatangelo,L, Lauro,R, Molinaro,M, Giaccari,A, Bouche,M: Transgenic mice with dominant negative PKC-theta in skeletal muscle: a new model of insulin resistance and obesity. *J Cell Physiol* 196:89-97, 2003

66. Dey,D, Basu,D, Roy,SS, Bandyopadhyay,A, Bhattacharya,S: Involvement of novel PKC isoforms in FFA induced defects in insulin signaling. *Mol Cell Endocrinol* 246:60-64, 2006

67. Shulman,GI: Cellular mechanisms of insulin resistance in humans. *Am J Cardiol* 84:3J-10J, 1999

68. de,AC, Teruel,T, Hernandez,R, Lorenzo,M: Tumor necrosis factor alpha produces insulin resistance in skeletal muscle by activation of inhibitor kappaB kinase in a p38 MAPK-dependent manner. *J Biol Chem* 279:17070-17078, 2004

69. Menzaghi,C, Plengvidhya,N, Ma,X, Warram,JH, Shoelson,SE, Doria,A: Genetic variability in insulin action inhibitor Ikkbeta (IKBKB) does not play a major role in the development of type 2 diabetes. *J Clin Endocrinol Metab* 87:1894-1897, 2002

70. Buchkovich,NJ, Yu,Y, Zampieri,CA, Alwine,JC: The TORrid affairs of viruses: effects of mammalian DNA viruses on the PI3K-Akt-mTOR signalling pathway. *Nat Rev Microbiol* 6:266-275, 2008

71. Nave,BT, Ouwens,M, Withers,DJ, Alessi,DR, Shepherd,PR: Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem J* 344 Pt 2:427-431, 1999

72. Takano,A, Usui,I, Haruta,T, Kawahara,J, Uno,T, Iwata,M, Kobayashi,M: Mammalian target of rapamycin pathway regulates insulin signaling via subcellular redistribution of insulin receptor substrate 1 and integrates nutritional signals and metabolic signals of insulin. *Mol Cell Biol* 21:5050-5062, 2001

73. Fraenkel,M, Ketzinel-Gilad,M, Ariav,Y, Pappo,O, Karaca,M, Castel,J, Berthault,MF, Magnan,C, Cerasi,E, Kaiser,N, Leibowitz,G: mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. *Diabetes* 57:945-957, 2008

74. Kimball,SR: The role of nutrition in stimulating muscle protein accretion at the molecular level. *Biochem Soc Trans* 35:1298-1301, 2007

75. Cheng,SW, Fryer,LG, Carling,D, Shepherd,PR: Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status. *J Biol Chem* 279:15719-15722, 2004

76. Vander,HE, Lee,SI, Bandhakavi,S, Griffin,TJ, Kim,DH: Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. *Nat Cell Biol* 9:316-323, 2007

77. Pullen,N, Thomas,G: The modular phosphorylation and activation of p70s6k. *FEBS Lett* 410:78-82, 1997

78. Kimura,N, Tokunaga,C, Dalal,S, Richardson,C, Yoshino,K, Hara,K, Kemp,BE, Witters,LA, Mimura,O, Yonezawa,K: A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. *Genes Cells* 8:65-79, 2003

79. Tremblay,F, Gagnon,A, Veilleux,A, Sorisky,A, Marette,A: Activation of the mammalian target of rapamycin pathway acutely inhibits insulin signaling to Akt and glucose transport in 3T3-L1 and human adipocytes. *Endocrinology* 146:1328-1337, 2005

80. Berg,CE, Lavan,BE, Rondinone,CM: Rapamycin partially prevents insulin resistance induced by chronic insulin treatment. *Biochem Biophys Res Commun* 293:1021-1027, 2002

81. Drummond,MJ, Bell,JA, Fujita,S, Dreyer,HC, Glynn,EL, Volpi,E, Rasmussen,BB: Amino acids are necessary for the insulin-induced activation of mTOR/S6K1 signaling and protein synthesis in healthy and insulin resistant human skeletal muscle. *Clin Nutr* 2008

82. Harrington,LS, Findlay,GM, Gray,A, Tolkacheva,T, Wigfield,S, Rebholz,H, Barnett,J, Leslie,NR, Cheng,S, Shepherd,PR, Gout,I, Downes,CP, Lamb,RF: The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J Cell Biol* 166:213-223, 2004

83. Krssak,M, Falk,PK, Dresner,A, DiPietro,L, Vogel,SM, Rothman,DL, Roden,M, Shulman,GI: Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* 42:113-116, 1999

84. Perseghin,G, Scifo,P, De,CF, Pagliato,E, Battezzati,A, Arcelloni,C, Vanzulli,A, Testolin,G, Pozza,G, Del,MA, Luzi,L: Intramyocellular triglyceride content is a determinant of in vivo insulin resistance in humans: a 1H-13C nuclear magnetic resonance spectroscopy assessment in offspring of type 2 diabetic parents. *Diabetes* 48:1600-1606, 1999

85. Randle,PJ: Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes Metab Rev* 14:263-283, 1998

86. Dimopoulos,N, Watson,M, Sakamoto,K, Hundal,HS: Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells. *Biochem J* 399:473-481, 2006

87. Lang,CH: Elevated plasma free fatty acids decrease basal protein synthesis, but not the anabolic effect of leucine, in skeletal muscle. *Am J Physiol Endocrinol Metab* 291:E666-E674, 2006

88. King M: Relevance of AMPK to Type 2 Diabetes. *Medical Biochemistry*11-17, 2007

89. Kahn,BB, Alquier,T, Carling,D, Hardie,DG: AMP-activated protein kinase: ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab* 1:15-25, 2005

90. Xiao,B, Heath,R, Saiu;P, Leiper,FC, Leone,P, Jing,C, Walker,PA, Haire,L, Eccleston,JF, Davis,CT, Martin,SR, Carling,D, Gamblin,SJ: Structural basis for AMP binding to mammalian AMP-activated protein kinase. *Nature* 449:496-500, 2007

91. Carling,D: The AMP-activated protein kinase cascade--a unifying system for energy control. *Trends Biochem Sci* 29:18-24, 2004

92. Woods,A, Cheung,PC, Smith,FC, Davison,MD, Scott,J, Beri,RK, Carling,D: Characterization of AMP-activated protein kinase beta and gamma subunits. Assembly of the heterotrimeric complex in vitro. *J Biol Chem* 271:10282-10290, 1996

93. Nilsson,EC, Long,YC, Martinsson,S, Glund,S, Garcia-Roves,P, Svensson,LT, Andersson,L, Zierath,JR, Mahlapuu,M: Opposite transcriptional regulation in skeletal muscle of AMP-activated protein kinase gamma3 R225Q transgenic versus knock-out mice. *J Biol Chem* 281:7244-7252, 2006

94. Hou,X, Xu,S, Maitland-Toolan,KA, Sato,K, Jiang,B, Ido,Y, Lan,F, Walsh,K, Wierzbicki,M, Verbeuren,TJ, Cohen,RA: SIRT1 regulates hepatocyte lipid metabolism through activating AMP-activated protein kinase. *J Biol Chem* 2008

95. Wang,W, Yang,X, Lopez,dS, I, Carling,D, Gorospe,M: Increased AMP:ATP ratio and AMP-activated protein kinase activity during cellular senescence linked to reduced HuR function. *J Biol Chem* 278:27016-27023, 2003

96. Mitchelhill,KI, Michell,BJ, House,CM, Stapleton,D, Dyck,J, Gamble,J, Ullrich,C, Witters,LA, Kemp,BE: Posttranslational modifications of the 5'-AMP-activated protein kinase beta1 subunit. *J Biol Chem* 272:24475-24479, 1997

97. Pang,T, Zhang,ZS, Gu,M, Qiu,BY, Yu,LF, Cao,PR, Shao,W, Su,MB, Li,JY, Nan,FJ, Li,J: Small molecule antagonizes autoinhibition and activates AMP-activated protein kinase in cells. *J Biol Chem* 283:16051-16060, 2008

98. Xie,M, Zhang,D, Dyck,JR, Li,Y, Zhang,H, Morishima,M, Mann,DL, Taffet,GE, Baldini,A, Khoury,DS, Schneider,MD: A pivotal role for endogenous TGF-beta-activated kinase-1 in the LKB1/AMP-activated protein kinase energy-sensor pathway. *Proc Natl Acad Sci U S A* 103:17378-17383, 2006

99. Breen,DM, Sanli,T, Giacca,A, Tsiani,E: Stimulation of muscle cell glucose uptake by resveratrol through sirtuins and AMPK. *Biochem Biophys Res Commun* 2008

100. Park,CE, Kim,MJ, Lee,JH, Min,BI, Bae,H, Choe,W, Kim,SS, Ha,J: Resveratrol stimulates glucose transport in C2C12 myotubes by activating AMP-activated protein kinase. *Exp Mol Med* 39:222-229, 2007

101. Jorgensen,SB, Viollet,B, Andreelli,F, Frosig,C, Birk,JB, Schjerling,P, Vaulont,S, Richter,EA, Wojtaszewski,JF: Knockout of the alpha2 but not alpha1 5'-AMP-activated protein kinase isoform abolishes 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranosidebut not contraction-induced glucose uptake in skeletal muscle. *J Biol Chem* 279:1070-1079, 2004

102. Wenzel,E, Somoza,V: Metabolism and bioavailability of trans-resveratrol. *Mol Nutr Food Res* 49:472-481, 2005

103. Billes,F, Mohammed-Ziegler,I, Mikosch,H, Tyihak,E: Vibrational spectroscopy of resveratrol. *Spectrochim Acta A Mol Biomol Spectrosc* 2006

104. Crowell,JA, Korytko,PJ, Morrissey,RL, Booth,TD, Levine,BS: Resveratrol-associated renal toxicity. *Toxicol Sci* 82:614-619, 2004

105. Ector B,MJHC&CM: Resveratrol concentrations in muscadine berries, juice, pomace, purees, seeds, and wine. *American Journal of Viticulture*57-62, 2007

106. Caruso,F, Tanski,J, Villegas-Estrada,A, Rossi,M: Structural basis for antioxidant activity of trans-resveratrol: ab initio calculations and crystal and molecular structure. *J Agric Food Chem* 52:7279-7285, 2004

107. Vitrac,X, Desmouliere,A, Brouillaud,B, Krisa,S, Deffieux,G, Barthe,N, Rosenbaum,J, Merillon,JM: Distribution of [14C]-trans-resveratrol, a cancer chemopreventive polyphenol, in mouse tissues after oral administration. *Life Sci* 72:2219-2233, 2003

108. Marier,JF, Vachon,P, Gritsas,A, Zhang,J, Moreau,JP, Ducharme,MP: Metabolism and disposition of resveratrol in rats: extent of absorption, glucuronidation, and enterohepatic recirculation evidenced by a linked-rat model. *J Pharmacol Exp Ther* 302:369-373, 2002

109. Hung,CF, Lin,YK, Huang,ZR, Fang,JY: Delivery of resveratrol, a red wine polyphenol, from solutions and hydrogels via the skin. *Biol Pharm Bull* 31:955-962, 2008

110. Boocock,DJ, Faust,GE, Patel,KR, Schinas,AM, Brown,VA, Ducharme,MP, Booth,TD, Crowell,JA, Perloff,M, Gescher,AJ, Steward,WP, Brenner,DE: Phase I dose escalation pharmacokinetic study in healthy volunteers of resveratrol, a potential cancer chemopreventive agent. *Cancer Epidemiol Biomarkers Prev* 16:1246-1252, 2007

111. Juan,ME, Vinardell,MP, Planas,JM: The daily oral administration of high doses of trans-resveratrol to rats for 28 days is not harmful. *J Nutr* 132:257-260, 2002

112. Jannin,B, Menzel,M, Berlot,JP, Delmas,D, Lancon,A, Latruffe,N: Transport of resveratrol, a cancer chemopreventive agent, to cellular targets: plasmatic protein binding and cell uptake. *Biochem Pharmacol* 68:1113-1118, 2004

113. Sengupta,B, Sengupta,PK: The interaction of quercetin with human serum albumin: a fluorescence spectroscopic study. *Biochem Biophys Res Commun* 299:400-403, 2002

114. Walle,T, Hsieh,F, DeLegge,MH, Oatis,JE, Jr., Walle,UK: High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab Dispos* 32:1377-1382, 2004

115. Belguendouz,L, Fremont,L, Gozzelino,MT: Interaction of transresveratrol with plasma lipoproteins. *Biochem Pharmacol* 55:811-816, 1998

116. Lancon,A, Delma,D, Osman,H, Thenot,JP, Jannin,B, Latruffe,N: Human hepatic cell uptake of resveratrol: involvement of both passive diffusion and carrier-mediated process. *Biochem Biophys Res Commun* 316:1132-1137, 2004

117. Kaldas,MI, Walle,UK, Walle,T: Resveratrol transport and metabolism by human intestinal Caco-2 cells. *J Pharm Pharmacol* 55:307-312, 2003

118. Baur,JA, Pearson,KJ, Price,NL, Jamieson,HA, Lerin,C, Kalra,A, Prabhu,VV, Allard,JS, Lopez-Lluch,G, Lewis,K, Pistell,PJ, Poosala,S, Becker,KG, Boss,O, Gwinn,D, Wang,M, Ramaswamy,S, Fishbein,KW, Spencer,RG, Lakatta,EG, Le,CD, Shaw,RJ, Navas,P, Puigserver,P, Ingram,DK, de,CR, Sinclair,DA: Resveratrol improves health and survival of mice on a high-calorie diet. *Nature* 444:337-342, 2006

119. Aggarwal,BB, Bhardwaj,A, Aggarwal,RS, Seeram,NP, Shishodia,S, Takada,Y: Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. *Anticancer Res* 24:2783-2840, 2004

120. Bradamante,S, Barenghi,L, Villa,A: Cardiovascular protective effects of resveratrol. *Cardiovasc Drug Rev* 22:169-188, 2004

121. de la Lastra,CA, Villegas,I: Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem Soc Trans* 35:1156-1160, 2007

122. Wang,Z, Zou,J, Huang,Y, Cao,K, Xu,Y, Wu,JM: Effect of resveratrol on platelet aggregation in vivo and in vitro. *Chin Med J (Engl)* 115:378-380, 2002

123. Lagouge,M, Argmann,C, Gerhart-Hines,Z, Meziane,H, Lerin,C, Daussin,F, Messadeq,N, Milne,J, Lambert,P, Elliott,P, Geny,B, Laakso,M, Puigserver,P, Auwerx,J: Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* 127:1109-1122, 2006

124. Finkel,T, Holbrook,NJ: Oxidants, oxidative stress and the biology of ageing. *Nature* 408:239-247, 2000

125. Martinez,J, Moreno,JJ: Effect of resveratrol, a natural polyphenolic compound, on reactive oxygen species and prostaglandin production. *Biochem Pharmacol* 59:865-870, 2000

126. Vieira de Almeida,LM, Pineiro,CC, Leite,MC, Brolese,G, Leal,RB, Gottfried,C, Goncalves,CA: Protective effects of resveratrol on hydrogen peroxide induced toxicity in primary cortical astrocyte cultures. *Neurochem Res* 33:8-15, 2008

127. Fremont,L: Biological effects of resveratrol. *Life Sci* 66:663-673, 2000

128. Brownson,DM, Azios,NG, Fuqua,BK, Dharmawardhane,SF, Mabry,TJ: Flavonoid effects relevant to cancer. *J Nutr* 132:3482S-3489S, 2002

129. Olas,B, Wachowicz,B, Saluk-Juszczak,J, Zielinski,T, Kaca,W, Buczynski,A: Antioxidant activity of resveratrol in endotoxin-stimulated blood platelets. *Cell Biol Toxicol* 17:117-125, 2001

130. Sgambato,A, Ardito,R, Faraglia,B, Boninsegna,A, Wolf,FI, Cittadini,A: Resveratrol, a natural phenolic compound, inhibits cell proliferation and prevents oxidative DNA damage. *Mutat Res* 496:171-180, 2001

131. Robb,EL, Page,MM, Wiens,BE, Stuart,JA: Molecular mechanisms of oxidative stress resistance induced by resveratrol: Specific and progressive induction of MnSOD. *Biochem Biophys Res Commun* 367:406-412, 2008

132. Kirimlioglu,V, Sozen,H, Turkoglu,S, Haberal,M: Protective effect of resveratrol, a red wine constituent polyphenol, on rats subjected to portal vein thrombosis. *Transplant Proc* 40:290-292, 2008

133. Liew,R, Stagg,MA, MacLeod,KT, Collins,P: The red wine polyphenol, resveratrol, exerts acute direct actions on guinea-pig ventricular myocytes. *Eur J Pharmacol* 519:1-8, 2005

134. Dekkers,DH, Bezstarosti,K, Gurusamy,N, Luijk,K, Verhoeven,AJ, Rijkers,EJ, Demmers,JA, Lamers,JM, Maulik,N, Das,DK: Identification by a differential proteomic approach of the induced stress and redox proteins by resveratrol in the normal and diabetic rat hearts. *J Cell Mol Med* 2008

135. Aubin,MC, Lajoie,C, Clement,R, Gosselin,H, Calderone,A, Perrault,LP: Female Rats Fed a High Fat Diet Were Associated with Vascular Dysfunction and Cardiac Fibrosis in the Absence of Overt Obesity and Hyperlipidemia: Therapeutic Potential of Resveratrol (trans-3,5,4'-Trihydroxystilbene). *J Pharmacol Exp Ther* 2008

136. Lekli,I, Szabo,G, Juhasz,B, Das,S, Das,M, Varga,E, Szendrei,L, Gesztelyi,R, Varadi,J, Bak,I, Das,DK, Tosaki,A: Protective mechanisms of resveratrol against ischemia-reperfusion-induced damage in hearts obtained from Zucker obese rats: the role of GLUT-4 and endothelin. *Am J Physiol Heart Circ Physiol* 294:H859-H866, 2008

137. Shankar,S, Siddiqui,I, Srivastava,RK: Molecular mechanisms of resveratrol (3,4,5-trihydroxy-trans-stilbene) and its interaction with TNF-related apoptosis inducing ligand (TRAIL) in androgen-insensitive prostate cancer cells. *Mol Cell Biochem* 304:273-285, 2007

138. Joe,AK, Liu,H, Suzui,M, Vural,ME, Xiao,D, Weinstein,IB: Resveratrol induces growth inhibition, S-phase arrest, apoptosis, and changes in biomarker expression in several human cancer cell lines. *Clin Cancer Res* 8:893-903, 2002

139. van Ginkel,PR, Darjatmoko,SR, Sareen,D, Subramanian,L, Bhattacharya,S, Lindstrom,MJ, Albert,DM, Polans,AS: Resveratrol Inhibits Uveal Melanoma Tumor Growth via Early Mitochondrial Dysfunction. *Invest Ophthalmol Vis Sci* 49:1299-1306, 2008

140. Schlachterman,A, Valle,F, Wall,KM, Azios,NG, Castillo,L, Morell,L, Washington,AV, Cubano,LA, Dharmawardhane,SF: Combined resveratrol, quercetin, and catechin treatment reduces breast tumor growth in a nude mouse model. *Transl Oncol* 1:19-27, 2008

141. Garvin,S, Ollinger,K, Dabrosin,C: Resveratrol induces apoptosis and inhibits angiogenesis in human breast cancer xenografts in vivo. *Cancer Lett* 231:113-122, 2006

142. Howitz,KT, Bitterman,KJ, Cohen,HY, Lamming,DW, Lavu,S, Wood,JG, Zipkin,RE, Chung,P, Kisielewski,A, Zhang,LL, Scherer,B, Sinclair,DA: Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. *Nature* 425:191-196, 2003

143. Wood,JG, Rogina,B, Lavu,S, Howitz,K, Helfand,SL, Tatar,M, Sinclair,D: Sirtuin activators mimic caloric restriction and delay ageing in metazoans. *Nature* 430:686-689, 2004

144. Milne,JC, Lambert,PD, Schenk,S, Carney,DP, Smith,JJ, Gagne,DJ, Jin,L, Boss,O, Perni,RB, Vu,CB, Bemis,JE, Xie,R, Disch,JS, Ng,PY, Nunes,JJ, Lynch,AV, Yang,H, Galonek,H, Israelian,K, Choy,W, Iffland,A, Lavu,S, Medvedik,O, Sinclair,DA, Olefsky,JM, Jirousek,MR, Elliott,PJ, Westphal,CH: Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* 450:712-716, 2007

145. Bass,TM, Weinkove,D, Houthoofd,K, Gems,D, Partridge,L: Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. *Mech Ageing Dev* 128:546-552, 2007

146. Kaeberlein,M, Kennedy,BK: Does resveratrol activate yeast Sir2 in vivo? *Aging Cell* 6:415-416, 2007

147. Borra,MT, Smith,BC, Denu,JM: Mechanism of human SIRT1 activation by resveratrol. *J Biol Chem* 280:17187-17195, 2005

148. de Ruijter,AJ, van Gennip,AH, Caron,HN, Kemp,S, van Kuilenburg,AB: Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* 370:737-749, 2003

149. Wade,PA: Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. *Hum Mol Genet* 10:693-698, 2001

150. li-Youcef,N, Lagouge,M, Froelich,S, Koehl,C, Schoonjans,K, Auwerx,J: Sirtuins: the 'magnificent seven', function, metabolism and longevity. *Ann Med* 39:335-345, 2007

151. Jin,YH, Kim,YJ, Kim,DW, Baek,KH, Kang,BY, Yeo,CY, Lee,KY: Sirt2 interacts with 14-3-3 beta/gamma and down-regulates the activity of p53. *Biochem Biophys Res Commun* 2008

152. North,BJ, Verdin,E: Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome Biol* 5:224, 2004

153. Kaeberlein,M, Kirkland,KT, Fields,S, Kennedy,BK: Sir2-independent life span extension by calorie restriction in yeast. *PLoS Biol* 2:E296, 2004

154. Zhang,J: The direct involvement of SirT1 in insulin-induced insulin receptor substrate-2 tyrosine phosphorylation. *J Biol Chem* 282:34356-34364, 2007

155. Tissenbaum,HA, Guarente,L: Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. *Nature* 410:227-230, 2001

156. Rogina,B, Helfand,SL: Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A* 101:15998-16003, 2004

157. Moynihan,KA, Grimm,AA, Plueger,MM, Bernal-Mizrachi,E, Ford,E, Cras-Meneur,C, Permutt,MA, Imai,S: Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. *Cell Metab* 2:105-117, 2005

158. Baur,JA, Sinclair,DA: Therapeutic potential of resveratrol: the in vivo evidence. *Nat Rev Drug Discov* 5:493-506, 2006

159. Rodgers,JT, Lerin,C, Haas,W, Gygi,SP, Spiegelman,BM, Puigserver,P: Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* 434:113-118, 2005

160. Yeung,F, Hoberg,JE, Ramsey,CS, Keller,MD, Jones,DR, Frye,RA, Mayo,MW: Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. *EMBO J* 23:2369-2380, 2004

161. Brooks,CL, Gu,W: p53 Activation: a case against Sir. *Cancer Cell* 13:377-378, 2008

162. Dryden,SC, Nahhas,FA, Nowak,JE, Goustin,AS, Tainsky,MA: Role for human SIRT2 NAD-dependent deacetylase activity in control of mitotic exit in the cell cycle. *Mol Cell Biol* 23:3173-3185, 2003

163. Inoue,T, Hiratsuka,M, Osaki,M, Yamada,H, Kishimoto,I, Yamaguchi,S, Nakano,S, Katoh,M, Ito,H, Oshimura,M: SIRT2, a tubulin deacetylase, acts to block the entry to chromosome condensation in response to mitotic stress. *Oncogene* 26:945-957, 2007

164. Shi,T, Wang,F, Stieren,E, Tong,Q: SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes. *J Biol Chem* 280:13560-13567, 2005

165. Haigis,MC, Mostoslavsky,R, Haigis,KM, Fahie,K, Christodoulou,DC, Murphy,AJ, Valenzuela,DM, Yancopoulos,GD, Karow,M, Blander,G, Wolberger,C, Prolla,TA, Weindruch,R, Alt,FW, Guarente,L: SIRT4 inhibits glutamate dehydrogenase and opposes the effects of calorie restriction in pancreatic beta cells. *Cell* 126:941-954, 2006

166. Mostoslavsky,R, Chua,KF, Lombard,DB, Pang,WW, Fischer,MR, Gellon,L, Liu,P, Mostoslavsky,G, Franco,S, Murphy,MM, Mills,KD, Patel,P, Hsu,JT, Hong,AL, Ford,E, Cheng,HL, Kennedy,C, Nunez,N, Bronson,R, Frendewey,D, Auerbach,W, Valenzuela,D, Karow,M, Hottiger,MO, Hursting,S, Barrett,JC, Guarente,L, Mulligan,R, Demple,B, Yancopoulos,GD, Alt,FW: Genomic instability and aging-like phenotype in the absence of mammalian SIRT6. *Cell* 124:315-329, 2006

167. Grummt,I, Pikaard,CS: Epigenetic silencing of RNA polymerase I transcription. *Nat Rev Mol Cell Biol* 4:641-649, 2003

168. Ford,E, Voit,R, Liszt,G, Magin,C, Grummt,I, Guarente,L: Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes Dev* 20:1075-1080, 2006

169. Mai,A, Massa,S, Lavu,S, Pezzi,R, Simeoni,S, Ragno,R, Mariotti,FR, Chiani,F, Camilloni,G, Sinclair,DA: Design, synthesis, and biological evaluation of sirtinol analogues as class III histone/protein deacetylase (Sirtuin) inhibitors. *J Med Chem* 48:7789-7795, 2005

170. Denu,JM: The Sir 2 family of protein deacetylases. *Curr Opin Chem Biol* 9:431-440, 2005

171. Yang,T, Sauve,AA: NAD metabolism and sirtuins: metabolic regulation of protein deacetylation in stress and toxicity. *AAPS J* 8:E632-E643, 2006

172. Sun,C, Zhang,F, Ge,X, Yan,T, Chen,X, Shi,X, Zhai,Q: SIRT1 Improves Insulin Sensitivity under Insulin-Resistant Conditions by Repressing PTP1B. *Cell Metab* 6:307-319, 2007

173. Su,HC, Hung,LM, Chen,JK: Resveratrol, a red wine antioxidant, possesses an insulin-like effect in streptozotocin-induced diabetic rats. *Am J Physiol Endocrinol Metab* 290:E1339-E1346, 2006

174. Alkhateeb,H, Chabowski,A, Glatz,JF, Luiken,JF, Bonen,A: Two phases of palmitate-induced insulin resistance in skeletal muscle: impaired GLUT4 translocation is followed by a reduced GLUT4 intrinsic activity. *Am J Physiol Endocrinol Metab* 293:E783-E793, 2007

175. Dey,D, Pal,BC, Biswas,T, Roy,SS, Bandyopadhyay,A, Mandal,SK, Giri,BB, Bhattacharya,S: A Lupinoside prevented fatty acid induced inhibition of insulin sensitivity in 3T3 L1 adipocytes. *Mol Cell Biochem* 300:149-157, 2007

176. Wilding,JP: The importance of free fatty acids in the development of Type 2 diabetes. *Diabet Med* 24:934-945, 2007

177. Klip,A, Logan,WJ, Li,G: Hexose transport in L6 muscle cells. Kinetic properties and the number of [3H]cytochalasin B binding sites. *Biochim Biophys Acta* 687:265-280, 1982

178. Kim,JK, Kim,YJ, Fillmore,JJ, Chen,Y, Moore,I, Lee,J, Yuan,M, Li,ZW, Karin,M, Perret,P, Shoelson,SE, Shulman,GI: Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest* 108:437-446, 2001

179. Watt,MJ, Hevener,A, Lancaster,GI, Febbraio,MA: Ciliary neurotrophic factor prevents acute lipid-induced insulin resistance by attenuating ceramide accumulation and phosphorylation of c-Jun N-terminal kinase in peripheral tissues. *Endocrinology* 147:2077-2085, 2006

180. Khamzina,L, Veilleux,A, Bergeron,S, Marette,A: Increased activation of the mammalian target of rapamycin pathway in liver and skeletal muscle of obese rats: possible involvement in obesity-linked insulin resistance. *Endocrinology* 146:1473-1481, 2005

181. Brozinick,JT, Jr., Birnbaum,MJ: Insulin, but not contraction, activates Akt/PKB in isolated rat skeletal muscle. *J Biol Chem* 273:14679-14682, 1998

182. Gescher,AJ, Steward,WP: Relationship between mechanisms, bioavailibility, and preclinical chemopreventive efficacy of resveratrol: a conundrum. *Cancer Epidemiol Biomarkers Prev* 12:953-957, 2003

183. Dann,SG, Selvaraj,A, Thomas,G: mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. *Trends Mol Med* 13:252-259, 2007

184. Fulco,M, Cen,Y, Zhao,P, Hoffman,EP, McBurney,MW, Sauve,AA, Sartorelli,V: Glucose restriction inhibits skeletal myoblast differentiation by activating SIRT1 through AMPK-mediated regulation of Nampt. *Dev Cell* 14:661-673, 2008

185. Giovannucci,E: Metabolic syndrome, hyperinsulinemia, and colon cancer: a review. *Am J Clin Nutr* 86:s836-s842, 2007

186. Tsukumo,DM, Carvalho-Filho,MA, Carvalheira,JB, Prada,PO, Hirabara,SM, Schenka,AA, Araujo,EP, Vassallo,J, Curi,R, Velloso,LA, Saad,MJ: Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* 56:1986-1998, 2007

187. Shi,H, Kokoeva,MV, Inouye,K, Tzameli,I, Yin,H, Flier,JS: TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 116:3015-3025, 2006